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Non-Alcoholic Steatohepatitis, Liver Cirrhosis and Hepatocellular Carcinoma: The Molecular Pathways

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Abstract

Non-alcoholic steatohepatitis (NASH) is growing into global problem, mainly due to NASH-induced cirrhosis and hepatocellular carcinoma (HCC), that can develop either subsequently to cirrhosis or preceding it. In addition, NASH-induced cirrhosis constitutes a significant fraction of cases diagnosed as cryptogenic cirrhosis. Thus, there is a need for deeper understanding of the molecular basis, leading to liver steatosis, then—to the associated inflammation seen in NASH, loss of liver architecture and cirrhosis, followed or paralleled by carcinogenesis and HCC. Insulin resistance, increased hepatic iron level, and certain cytokines, including TNF- α and IL-6 derived from extrahepatic adipose tissues, can trigger the chain of events. The imbalance between leptin and adiponectin is important as well. These markers remain important during the whole course from NASH through liver cirrhosis to HCC. The molecular pathogenesis substantiates treatment: hypertriglyceridemia can be lowered by low calorie diet; mTOR complex can become inhibited by physical activity and metformin; cholesterol synthesis, RAF/MAPK1/ERK and p21 pathway by statins; inflammation by pentoxifyllin, and kinases (in HCC) by sorafenib. Bidirectional regulation of telomere attrition, senescence and p21 pathway, restoration of wild-type p53 activity and regulation of miRNA network represent attractive future treatment options. Focusing on relevant molecular pathways allows deeper understanding of NASH pathogenesis, leading to identification of predictive markers and treatment targets.

Keywords: non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, liver cirrhosis, cryptogenic cirrhosis, hepatocellular carcinoma

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical and pathological entity with features that resemble alcohol-induced liver steatosis, but, by the definition, it occurs in patients with little or no history of alcohol consumption. NAFLD is subdivided into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). It encompasses a histological spectrum that ranges from fat accumulation in hepatocytes without concomitant inflammation or fibrosis (simple hepatic steatosis, NAFL) to hepatic steatosis with a necroinflammatory component (inflammation-induced apoptosis in hepatocytes) that may or may not have associated fibrosis. The latter condition, referred to as non-alcoholic steatohepatitis (NASH), can lead to NASH-induced liver cirrhosis (**Figure 1**). In addition, NASH is now recognised as the main cause of cryptogenic cirrhosis [1], as sequential association has been demonstrated in up to 75% of cryptogenic cirrhosis cases (see also Section 3 for detailed discussion of the relationships between NASH and cryptogenic cirrhosis). Liver cirrhosis may further lead to hepatocellular carcinoma (HCC), the most common primary liver cancer known for its poor clinical outcome and limited therapeutic options. Although previously it was considered that risk of HCC is limited to cirrhotic patients [2], a significant fraction of NASH-associated HCC develops in liver showing none or mild fibrosis. The association between NAFLD/NASH and increased HCC risk is supported by strong epidemiologic evidence.

In the year 2010, the annual incidence of HCC in the population of the USA was at least 6 per 100,000. The mortality rate was almost identical to the incidence underscoring the serious prognosis [3]. Patients with NAFLD/NASH are subjected to an increased lifetime risk of HCC. In a 16-year follow-up study, the standardised incidence ratio of HCC in patients with NAFLD/NASH was 4.4 [4]. In a recent global meta-analysis, the HCC incidence among NAFLD patients reached 0.44 (range, 0.29–0.66) per 1000 person-years [5]. The HCC-related mortality rates among NAFLD patients range from 0.25 to 2.3% over 8.3 and 13.7 years of follow-up, respectively [5, 6]. NAFLD/NASH-associated HCC is believed to be the leading cause of obesity-related cancer deaths in middle-aged men in the USA [4]. Consistently, the proportion of HCC related to NAFLD/NASH is increasing worldwide and is reported to range between 4 and 22% in Western countries [7]. Although the exact burden of HCC associated with NAFLD/NASH still remains uncertain, it seems evident that NAFLD and NASH will become the most common causative/risk factors for HCC, surpassing viral or alcohol-related cirrhosis in the future [7]. In the USA, the number of NAFLD-associated HCC cases is annually growing (2004–2009) for 9% [8], while decreased burden of viral hepatitis-induced HCC might be expected due to the achievements in antiviral treatment targeting hepatitis C virus [9].

NAFLD is the major hepatic manifestation of obesity and associated metabolic conditions. The epidemiology of NAFLD mirrors the recent spread of obesity and diabetes. With increasing prevalence of these conditions, NAFLD has become the most common liver disorder in USA [10] and other Western industrialised countries, facing high occurrence of the major risk factors for NAFLD, namely, central obesity, type 2 diabetes mellitus, dyslipidemia and metabolic syndrome [11]. In a recent meta-analysis of 86 studies, comprising 8,515,431 persons from 22 countries, the global prevalence of NAFLD was 25.24% (95% confidence interval [CI], 22.10–28.65) showing the highest occurrence in the Middle East and South America and the lowest in Africa [5].

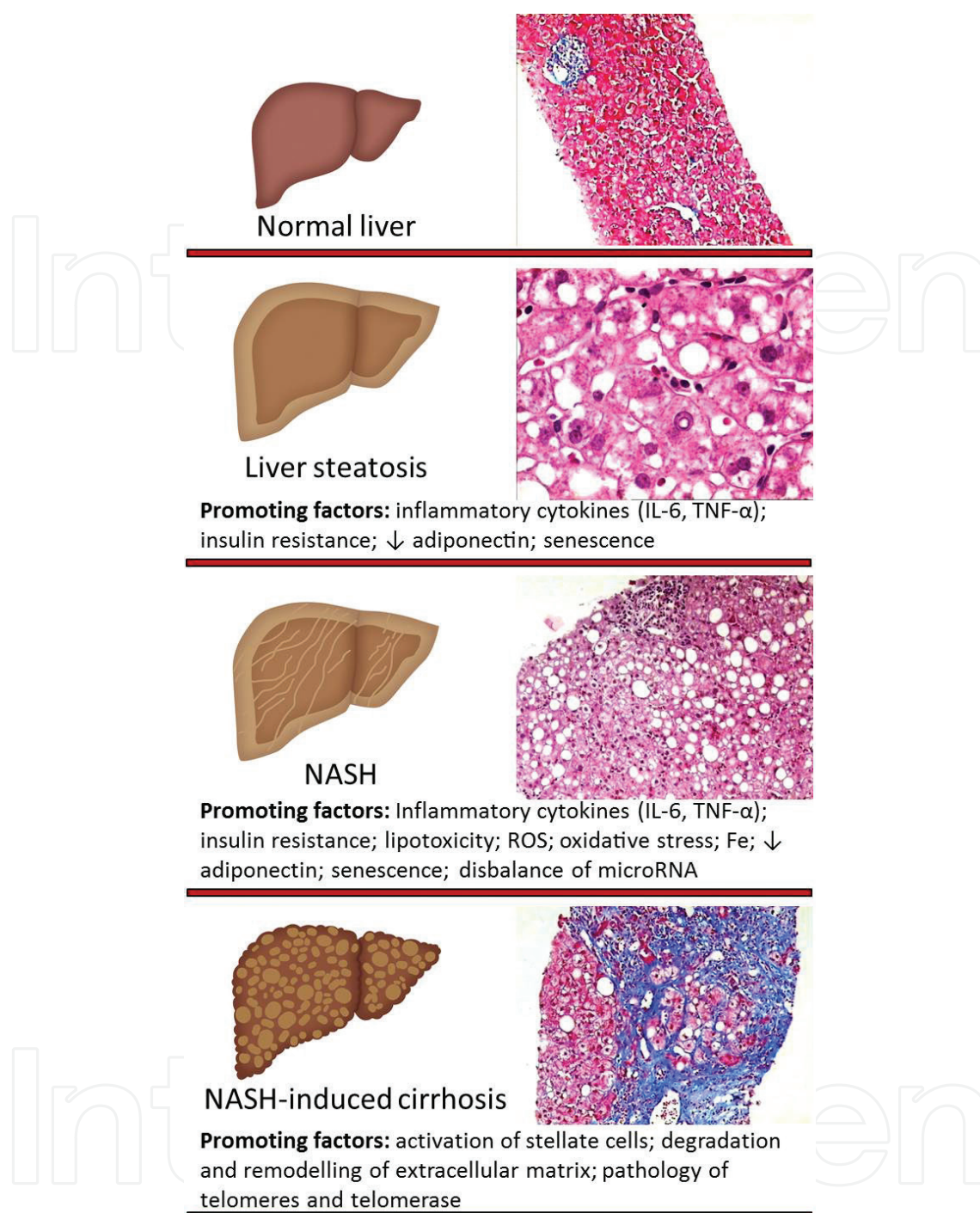


Figure 1. Progression of NAFLD. Abbreviations: IL, interleukin; TNF, tumour necrosis factor; ROS, reactive oxygen species; Fe, accumulation of iron compounds.

Thus, 90% of patients suffering from morbid obesity (defined as having body mass index 40 kg/m² or higher) and 74% patients affected by diabetes mellitus develop NAFLD. In addition, NAFLD has been observed even in non-obese, non-diabetic patients who have increased insulin levels in blood and resistance to insulin action. Consequently, NAFLD affects up to 20–30% of adults in Europe and 46% in the USA: a tremendously high prevalence for a condition that can cause any significant complications [9, 10].

Most patients are diagnosed with NAFLD in their 40s or 50s. Studies vary in regard to the gender distribution of NAFLD, with some suggesting that it is more common in women and others suggesting more frequent occurrence in men [11, 12].

Since 1998, non-alcoholic fatty liver disease has been considered a condition with a “two-hit” course of pathogenesis, first proposed by Day and James [13], describing the role of lipid peroxidation in liver injury. The “first hit” is the development of hepatic steatosis. It was suggested that hepatic triglyceride accumulation increased the susceptibility of the liver to the “second injury hit” by inflammatory cytokines and/or adipokines, mitochondrial dysfunction and elevated oxidative stress that together promote steatohepatitis and fibrosis [14]. Alternatively, many factors may act simultaneously leading to the development of NAFLD: this hypothesis corresponds to the multihit model proposed by Tilg and Moschen [15].

Experimental and population studies have shown the links between NAFLD/NASH and development of HCC. However, the mechanisms by which NASH progresses to HCC are only beginning to be elucidated [14]. NASH is the most rapidly growing risk for liver transplantation because of HCC. Wong et al. in their study included 61,868 patients over the period 2002–2012 and found that the proportion of NASH-related HCC increased from 8.3 to 13.5%, an increase of near 63% [16].

This increase is alarming as HCC already is the fifth most frequently diagnosed cancer and the second leading oncologic death cause worldwide [17], with increasing incidence and mortality rates in Europe [18]. Thus it is crucial to analyse molecular pathways involved in NASH-induced cirrhosis and HCC carcinogenesis. Focusing on the molecular events involved in pathogenetic chain of events from NASH to liver cirrhosis and HCC would provide not only better theoretical understanding of liver diseases preceding and following cirrhosis but would also allow to recognise predictive markers and treatment targets before HCC development.

2. Common pathogenetic mechanisms of NAFLD

Hepatic steatosis or excessive triglyceride accumulation in the liver is a prerequisite to the histological diagnosis of NAFLD. Several mechanisms may lead to steatosis, including (1) increased fat supply because of high-fat diet or excess lipolysis in adipose tissues, which increase free fatty acid (FFA) level; (2) decreased fat export in the form of very low density lipoprotein-triglyceride complex, secondary to either reduced synthesis of the relevant proteins or compromised excretion; (3) decreased or impaired β -oxidation of FFA to adenosine triphosphate and (4) increased hepatic synthesis of fatty acids through *de novo* lipogenesis [1, 19]. Free fatty acid delivery to the liver accounts for almost two-thirds of its lipid accumulation. *De novo* lipogenesis therefore only contributes to the accumulation of hepatic fat in case of NAFLD [15].

The molecular mechanisms responsible for the accumulation of fat in the liver are complex (**Figure 2**). Certain inflammatory cytokines, particularly those derived from extrahepatic

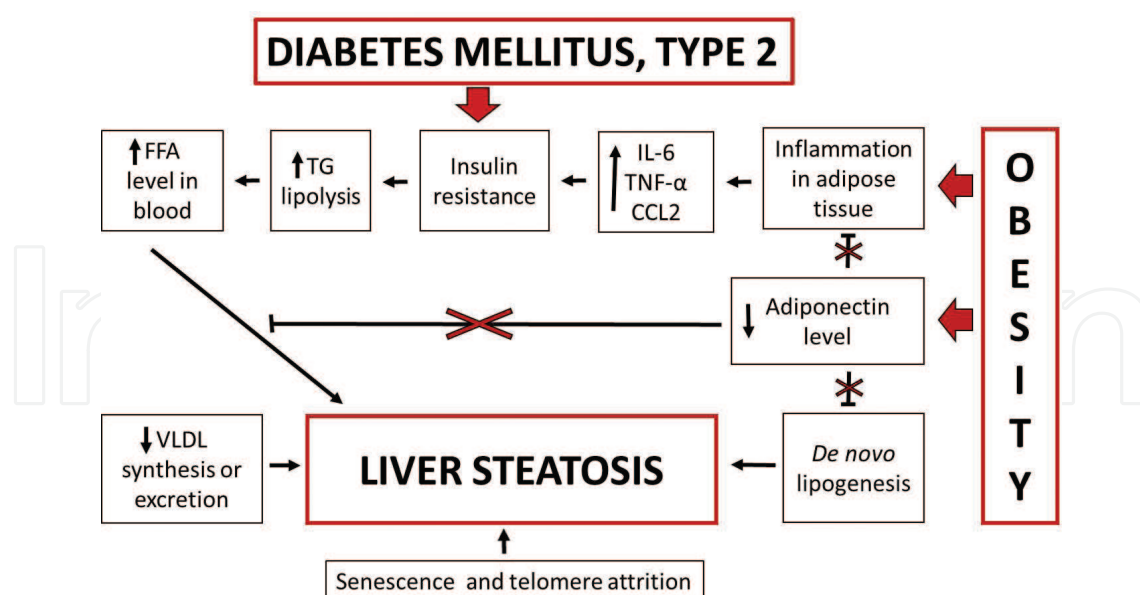


Figure 2. Pathogenesis of liver steatosis. Abbreviations: FFA, free fatty acids; TG, triglycerides; IL, interleukin; TNF, tumour necrosis factor; CCL2, CC motif chemokine ligand 2; VLDL, very low density lipoproteins.

adipose tissues, can trigger this process. Insulin resistance appears to be at the centre for the massive metabolic dysregulations that initiate and aggravate hepatic steatosis. At a certain point, the simple steatosis transforms to steatohepatitis in about 20–30% of NAFLD patients [19]. A major feature in the transition from NAFLD to NASH is the appearance of hepatic inflammation [14]. This breakthrough-like process is mediated by the interplay of multiple hit factors and is orchestrated by rich network of miRNAs [20]. Currently, a number of common pathogenetic mechanisms have been proposed and characterised for the transition from simple steatosis to NASH [19]. A summary of these mechanisms is shown in **Figure 3**.

2.1. Inflammation in peripheral adipose tissue

Hypoxia and death of rapidly expanding adipocytes are considered important initiating factors of adipose tissue inflammation in obesity [19]. During inflammation, typical cytokines like tumour necrosis factor (TNF)- α , interleukin (IL)-6 and CC motif chemokine ligand 2 (CCL2) are secreted by inflammatory cells infiltrating adipose tissue [21]. TNF- α was the first pro-inflammatory cytokine detected in adipose tissue. TNF- α and IL-6 are involved in the regulation of insulin resistance [19]. TNF- α and IL-6 induce insulin resistance in adipocytes, stimulating triglyceride lipolysis and fatty acid release into the circulation. CCL2 recruits macrophages to the adipose tissue, resulting in even higher local cytokine production and perpetuating the inflammatory cycle [19]. In the liver, increased expression of hepatic IL-6 correlates with higher degree of insulin resistance in patients with suspected NAFLD [1].

At the same time, extrahepatic adipocytes are compromised in their natural ability to secrete adiponectin, an anti-inflammatory adipokine that facilitates the normal partitioning of lipid

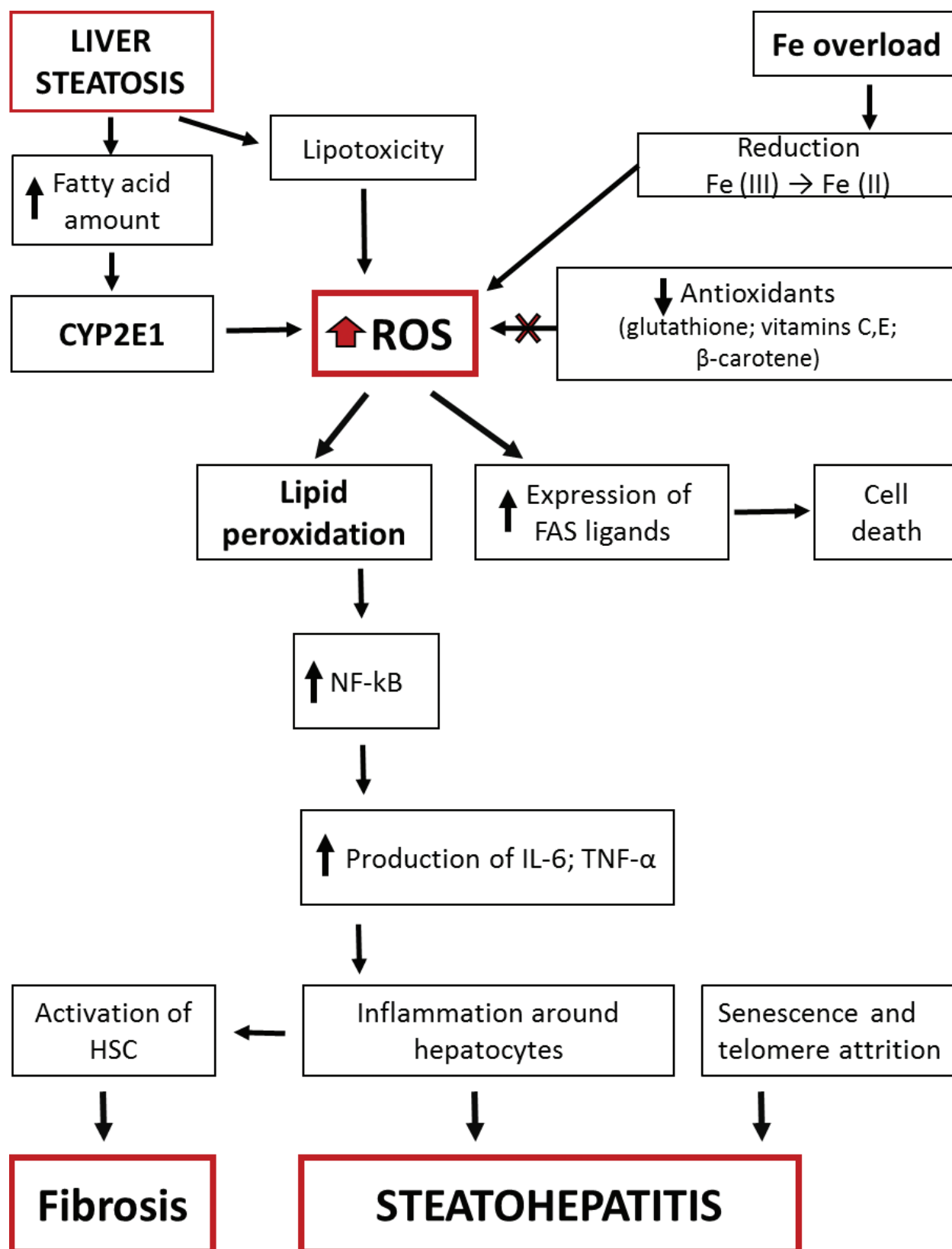


Figure 3. Pathogenesis of non-alcoholic steatohepatitis. Abbreviations: CYP2E1, cytochrome CYP2E1; ROS, reactive oxygen species; Fe, iron; NF-κB, nuclear factor kappaB; IL, interleukin; TNF, tumour necrosis factor; HSC, hepatic stellate cells.

to adipocytes for storage [19]. Adiponectin is a hormone secreted exclusively by adipose tissue. It has beneficial effects on lipid metabolism. In the liver, adiponectin is considered to have insulin-sensitising, anti-fibrogenic and anti-inflammatory properties by acting on hepatocytes, liver stellate cells and hepatic macrophages (Kupffer cells), respectively. Adiponectin suppresses the transportation of free fatty acids to the liver as well as gluconeogenesis and *de novo* synthesis of fats but enhances oxidation of FFAs [21]. The adiponectin-induced suppression of aldehyde oxidase and transforming growth factor has net anti-fibrotic effect [21], while decreased release of pro-inflammatory cytokines including TNF- α reduces inflammation [1]. Decreased levels of adiponectin result in loss of these protective metabolic, anti-fibrotic and anti-inflammatory effects.

Together, these abnormalities accentuate fat loss from adipocytes and promote ectopic fat accumulation [19].

2.2. Insulin resistance

Obesity and type 2 diabetes mellitus, both conditions associated with peripheral insulin resistance, are frequently diagnosed in patients affected by non-alcoholic fatty liver disease [12]. Evaluating patients suffering from diabetes mellitus, NAFLD was found in 74% of them in North American study, 70% in Italian population and 35–56% in Eastern countries. In Mexico, prevalence of NASH in diabetics was 18.5%. The prevalence of NAFLD in obese patients is 57–90% in Western and 10–80% in Eastern populations. NASH is present in 15–20% patients affected by obesity. The frequency of NASH is higher in those undergoing bariatric surgery and can reach 48–60% in USA men, 20–31% in USA females and up to 80% in Taiwan patients [9, 10, 12].

Insulin resistance has also been observed in NASH patients who are not obese and those who have normal glucose tolerance [1]; however, not all people with NAFLD have increased insulin resistance. NAFLD also cannot be considered as a cause for insulin resistance but rather as a consequence [19].

Resistance to the action of insulin results in important metabolic changes, including the turnover of lipids. It is characterised not only by increased circulating insulin levels but also by increased hepatic gluconeogenesis, impaired glucose uptake by muscle, enhanced peripheral lipolysis, increased triglyceride synthesis and increased hepatic uptake of fatty acids, as well as increased release of inflammatory cytokines from peripheral adipose tissues, which are the key factors promoting accumulation of liver fat and progression of hepatic steatosis [1, 19].

2.3. Lipotoxicity

The term “lipotoxicity” describes the deleterious effects of excess FFA and ectopic fat accumulation resulting in organ dysfunction and/or cellular death. In obesity, excessive food intake combined with high FFA output from insulin-resistant adipose tissue surpasses the storage and oxidative capacity of tissues such as skeletal muscle, liver, or pancreatic β -cells [22]. Long-chain saturated fatty acids, as well as free cholesterol derived from *de novo* synthesis can be harmful to hepatocytes. Free cholesterol accumulation leads to liver injury through

the activation of intracellular signalling pathways in Kupffer cells, liver stellate cells, and hepatocytes [19], ultimately promoting inflammation and fibrosis [23]. FFAs are redirected into noxious pathways of nonoxidative metabolism with intracellular accumulation of toxic metabolites. It is not TG accumulation *per se* that is uniquely hazardous, but rather the lipid-derived metabolites that trigger the development of reactive oxygen species (ROS) and activation of inflammatory pathways [22], including up-regulation of nuclear factor kappaB, production of TNF- α and IL-6 [24], and the subsequent inflammatory reaction in the liver [1].

2.4. Oxidative stress

In the context of increased supply of fatty acids to hepatocytes, oxidative stress can occur. It is attributable to the raised levels of reactive oxygen/nitrogen species and lipid peroxidation that are generated during free fatty acid metabolism in microsomes, peroxisomes, and mitochondria [19]. NAFLD and NASH-induced oxidative stress is partly regulated through cytochrome P450 2E1 (CYP2E1) as it metabolises C10–C20 fatty acids [14] that in turn produce hepatotoxic free oxygen radical species [1]. Peroxidation of plasma and intracellular membranes may cause direct cell necrosis/apoptosis and development of megamitochondria, while ROS-induced expression of Fas-ligand on hepatocytes may induce fratricidal cell death [19]. Recent studies support the idea that oxidative stress may be a primary cause of liver fat accumulation and subsequent liver injury [25], as well as ROS may play a part in fibrosis development. Lipid peroxidation and free oxygen radical species can also deplete antioxidant stores such as glutathione, vitamin E, beta-carotene, and vitamin C, rendering the liver susceptible to oxidative injury [1].

2.5. Increased hepatic iron concentration

The degree of liver fibrosis in nonalcoholic steatohepatitis shows correlation with the concentration of iron compounds in the hepatocytes. The underlying mechanism might involve the ferric-to-ferrous reduction (switch of trivalent Fe(III) to divalent Fe(II) compounds), resulting in simultaneous production of free oxygen radicals [1]. In addition, sinusoidal iron accumulation might also have a pathogenetic role in the progression of chronic liver diseases and development of hepatocellular carcinoma [26]. However, at least in Eastern populations, disturbances of iron metabolism are rarely observed in NAFLD patients [12]. In patients without iron overload, increased ferritin level in the blood may still be associated with insulin resistance and fatty liver [27].

2.6. MicroRNAs in NAFLD

MicroRNAs are small molecules of non-coding RNA that act as large-scale molecular switches. The pathogenetic chain of events in the transition to NAFL, NASH, and liver cirrhosis is richly regulated by miRNA network: it has been estimated that approximately 54 miRNAs regulate 107 genes involved in the development of NAFLD. The up-regulation of miR-26b and down-regulation of miR-26a decrease insulin sensitivity, while lower levels of miR-451 are associated with pro-inflammatory background. The up-regulation of miR-155 and miR-107 promotes fat accumulation in liver cells. Enhanced fibrosis is mediated by miR-21. Assessing

patients with NAFLD-associated liver fibrosis, at least 9 miRNAs are expressed in modified levels, including higher expression of miR-31, miR-182, miR-183, miR-224, and miR-150 as well as down-regulated levels of miR-17, miR-378i, miR-219a, and miR-590. In the progression of liver fibrosis, the normally high levels of miR-22 and miR-125b are suppressed. The miR-29 family showing anti-fibrotic action in many organs is also suppressed [20].

3. NASH-induced liver cirrhosis

Liver cirrhosis develops (**Table 1**) when simple steatosis progresses to steatohepatitis and then fibrosis [11]. The composition of the hepatic fibrosis is similar regardless of the cause of injury as it follows the paradigm for wound healing in other tissues, including skin, lung and kidney. Fibrosis occurs first in regions of most severe injury over several months to years of ongoing tissue damage [23, 28, 29].

Targets	Involved cells or molecules	Result
Stellate cells	Activated stellated cells are transformed to proliferating, fibrogenic and contractile myofibroblasts	Remodelling of the matrix
Macromolecules in the extracellular matrix	Collagens: the total collagen content increases 3- to 10-fold including an increase in fibril-forming collagens (i.e., types I, III, and IV) and some non-fibril forming collagens (types IV and VI). Glycoproteins: fibronectin, laminin, SPARC, osteonectin, tenascin, and von Willebrand factor Matrix-bound growth factors Glycosaminoglycans: perlecan, decorin, aggrecan, lumican, and fibromodulin Proteoglycans: shift from heparan sulphate-containing proteoglycans to those containing chondroitin and dermatan sulphates	The extracellular matrix switches from the normal low-density basement membrane-like matrix to the interstitial type
Degradation of extracellular matrix	Matrix metalloproteinase 2 Matrix metalloproteinase 9 Membrane-type metalloproteinase 1 and/or 2 Stromelysin 1	Disruption of normal matrix facilitates replacement by desmoplastic matrix

Table 1. The key structures in the development of liver cirrhosis.

Cryptogenic cirrhosis is the end stage of a chronic liver disease in which the underlying aetiology remains unknown after extensive clinical, serological and pathological evaluation [30, 31]. In different studies, 3–30% of liver cirrhosis cases have been attributed to the cryptogenic group [9]. Naturally, occasionally the diagnosis of cryptogenic cirrhosis is issued just due to lack of information despite the definition demanding complete investigation. Studying explanted livers of cirrhotic patients undergoing liver transplantation and having preoperative diagnosis of cryptogenic cirrhosis, specific cause was identified in 28.6% of cases. The relevant diagnoses included autoimmune hepatitis, sarcoidosis, primary biliary cirrhosis, sclerosing cholangitis, congenital hepatic fibrosis and Wilson's disease [32]. Other data/investigational methods can yield significant information as well. For instance, a significant fraction of cases initially diagnosed as cryptogenic liver cirrhosis can be associated with occult hepatitis B infection [33].

Recent evidence suggests that cryptogenic cirrhosis is strongly associated with development of HCC, while in a varying percentage (6.9–50%) of HCC, the underlying aetiology of liver disease cannot be determined. In a retrospective study of 641 HCC patients, cryptogenic cirrhosis was found in 44 (6.9%) cases, characterised also by more frequent occurrence of obesity and diabetes mellitus than in patients having history of chronic viral hepatitis and alcohol abuse. Considering the known association between obesity, diabetes and NASH, it was hypothesised that NASH is the precursor of cryptogenic cirrhosis and hepatocellular carcinoma [34].

At present, there is strong evidence that cryptogenic cirrhosis represents the end state of NASH at least in a fraction of patients. First, the progression of fibrosis in NASH is associated with gradual loss of fat vacuoles. Thus, the specific morphological changes would be burned out when the cirrhosis develops. Second, patients diagnosed with cryptogenic cirrhosis have high prevalence of metabolic changes as type 2 diabetes mellitus, obesity, or history of those disorders. If the history of preceding diabetes mellitus or obesity or liver biopsy revealing NAFLD is considered as the diagnostic criteria, 30–75% of cryptogenic cirrhosis cases can be retrospectively associated with NASH [9]. Third, due to growing awareness of the entity of NASH-induced cirrhosis, direct evidence has been brought by data obtained in explanted livers. Cases that were clinically diagnosed as cryptogenic cirrhosis were reclassified as NAFLD (either cirrhosis or pre-cirrhotic stage) in 78.6% of cases [12, 35, 36].

In comparison with liver cirrhosis due to other aetiologies, NASH-induced cirrhosis is diagnosed in older patients. Higher cardiovascular mortality is observed, in addition to the classic complications of liver cirrhosis attributable to portal hypertension and oesophageal variceal bleeding, infections and renal failure [9].

In a population-based, large study, carried out in the United Kingdom, the following distribution of cirrhosis by the cause was found (in patients, diagnosed in 1987–2006): alcohol-induced, 56.1%; cryptogenic, 20.8%; attributable to viral hepatitis, 12.0%; autoimmune or metabolic (i.e., in this study—haemochromatosis or alpha-1-antitrypsin deficiency), 11.0% [37]. In a nationwide Danish study regarding 11,605 patients diagnosed with liver cirrhosis in 1977–1989, 61.7% of cases were alcohol-induced, 2.8%—attributable to primary biliary cirrhosis, 14.6%—related to chronic hepatitis (including autoimmune inflammation) and 20.9%—

non-specified [38]. Regarding the cause of cirrhosis in explanted livers, 48.6% were related to chronic viral hepatitis (31.1% to HCV and 15.9% to HBV, 1.6% to HCV and HBV coinfection), 23.1% to alcohol-induced liver damage and 16.7% to NAFLD [36]. The data on explanted livers may not reflect the true incidence of NASH-induced cirrhosis as NAFLD patients are less likely to receive transplant. The probability to receive liver transplant within 1 year is 40.5% in NAFLD, contrasting with 47% for hepatitis C or alcohol-induced cirrhosis. The difference is the result of several factors: contraindications due to morbid obesity, comorbidities, older physiologic age, impaired renal function as well as slower disease progression [9].

Thus, cryptogenic cirrhosis is a significant burden for health care systems. Patients undergoing liver transplantation for cryptogenic cirrhosis are subjected to higher postoperative mortality, lower cumulative 5- and 10-year survival and higher rate of chronic rejection [32]. NASH is the most rapidly growing indication for simultaneous liver and kidney transplantation. NASH and cryptogenic cirrhosis in patients having body mass index greater than 30 kg/m² constituted 6.3% in the years 2002–2003 but 19.2% in the years 2010–2011 [39].

As the liver becomes fibrotic, significant changes occur in the extracellular matrix (ECM) quantitatively and qualitatively. ECM refers to macromolecules that comprise the scaffolding of either normal or fibrotic liver. These include collagens, non-collagen glycoproteins, matrix-bound growth factors, glycosaminoglycans, proteoglycans and matricellular proteins. In case of fibrosis, the total collagen content increases 3- to 10-fold including an increase in fibril-forming collagens (i.e., types I, III and IV) and some non-fibril forming collagens (types IV and VI). Glycoproteins (fibronectin; laminin; secreted protein, acidic and rich in cysteine: SPARC; osteonectin; tenascin, and von Willebrand factor), proteoglycans and glycosaminoglycans (perlecan, decorin, aggrecan, lumican, and fibromodulin) also accumulate in cirrhotic liver. Particularly notable is the shift from heparan sulphate-containing proteoglycans to those containing chondroitin and dermatan sulphates. These processes represent a change in the type of ECM in subendothelial space from the normal low-density basement membrane-like matrix to the interstitial type.

The replacement of the low-density matrix with the interstitial type influences the function of hepatocytes, liver stellate cells, and endothelium of blood vessels: the microvilli disappear on the surface of liver parenchymal cells, and endothelium loses fenestrations precluding effective molecule exchange between blood and liver parenchyma. In addition, stellate cells undergo activation [23].

The hepatic stellate cell is the primary source of ECM in normal and fibrotic liver. Hepatic stellate cells, located in subendothelial space of Disse between hepatocytes and sinusoidal endothelial cells, represent one-third of the non-parenchymal population or approximately 15% of the total number of resident cells in normal liver. Stellate cells comprise a heterogeneous group of cells that are functionally and anatomically similar but differ in their expression of cytoskeletal filaments, retinoid content, and potential for activation. Stellate cells with fibrogenic potential are not confined to liver and have been identified in other organs such as the pancreas, where they contribute to desmoplasia in chronic pancreatitis and carcinoma. Hepatic stellate cell activation is the common pathway leading to hepatic fibrosis. During activation, stellate cells undergo a transition from a quiescent vitamin A-rich cell into

proliferating, fibrogenic, and contractile myofibroblasts [23], which have strong ability to secrete collagen and migrate to the area of necrosis and inflammation [40]. Proliferation of stellate cells occurs predominantly in regions of greatest injury.

Considering liver fibrosis, the balance between synthesis and degradation of extracellular matrix also is of importance as enhanced destruction of the normal matrix in the space between hepatocytes and endothelial cells leads to accumulation of dense scar tissue. Degradation occurs through the actions of at least four enzymes: matrix metalloproteinase (MMP) 2 and MMP9, which degrade type IV collagen; membrane-type metalloproteinase 1 or 2, which activate latent MMP2 and stromelysin 1, which degrades proteoglycans and glycoproteins and activates latent collagenases. Stellate cells are the principal source of MMP2 and stromelysin. Activation of latent MMP2 may require interaction with hepatocytes. Markedly increased expression of MMP2 is a characteristic of cirrhosis. MMP9 is secreted locally by Kupffer cells. Disruption of the normal liver matrix is also a prerequisite for tumour invasion and stromal desmoplasia.

The cytochrome CYP2E1 may have an important role in the generation of reactive oxygen species that stimulate liver stellate cells. Cultured hepatic stellate cells grown in the presence of CYP2E1-expressing cells increase the production of collagen, an effect prevented by antioxidants or a CYP2E1 inhibitor. These data suggest that the CYP2E1-derived reactive oxygen species are responsible for the increased collagen production. Such findings may help to explain the pathogenesis of liver injury in alcoholic liver disease since CYP2E1 is alcohol inducible. As noted above, reactive oxygen species are generated through lipid peroxidation from hepatocytes, macrophages, stellate cells, and inflammatory cells. In alcoholic or non-alcoholic steatohepatitis, ROS generation in hepatocytes results from induction of cytochrome P450 2E1, leading to pericentral (zone 3) injury. Also, oxidase of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) mediates fibrogenic activation of hepatic stellate cells, as well as of Kupffer cells or resident liver macrophages through generation of oxidative stress. Increasing knowledge about NADPH oxidase isoforms and their cell-specific activities is leading to their emergence as a therapeutic target [23].

Pathology of telomeres and the related molecular events represent another key mechanism that is associated both with induction of liver steatosis and progression of NAFLD [41]. Telomerase mutations can accelerate progression of chronic liver disease to cirrhosis [42]. Missense mutations in telomerase reverse transcriptase hTERT are found more frequently in cirrhosis regardless of aetiology [41]. Thus, missense mutations were observed in 7% of cirrhotic patients in USA [43]. Functional mutations were identified in 3% of German patients affected by cirrhosis [44].

Telomeres are repeated, short DNA sequences (in humans—TTAGGG) located at the chromosome end. These structures prevent chromosomal end-to-end fusion as well as protect the coding DNA from progressive loss at mitosis. During each mitosis, the DNA polymerase complex cannot replicate the terminal 5' end of the lagging strand. Consequently, the chromosomal end is lost. Due to the presence of telomeres, this loss is limited to telomeres. However, the telomeres shorten in each mitosis. Telomere attrition is especially marked in chronic diseases associated with increased cell loss and proliferation. When they become critically short, cellular

ageing *s.* senescence and apoptosis follows. To ensure the unlimited proliferation of cancer, malignant cells maintain telomere length via different mechanisms. The most significant ones include telomerase reverse transcriptase hTERT, its RNA template: telomerase RNA component hTERC, the hTERC-protecting and stabilising dyskerin complex (consisting of four nucleolar proteins) and shelterin complex, including six proteins [41].

NAFLD is characterised by telomere shortening and increased cellular senescence in comparison to healthy controls [45]. The changes in telomeres represent an important mechanism in the transition to liver cirrhosis. However, dual effects are observed. In progressing chronic liver disease, cellular senescence enhances the loss of parenchyma, limiting the replicative potential of hepatocytes. In contrast, in advanced liver damage, the ageing of stellate cells stops the remodelling and thus, the further progression of fibrosis. Still another prognostic aspect can be involved regarding HCC development: senescent stellate cells can promote carcinogenesis by secreting pro-carcinogenic mediators. These changes are described as the senescence-associated secretory program [41]. The extent of fibrosis in NAFLD is associated with p21 protein representing another molecular regulator of cellular senescence [41].

Although shorter telomeres are considered a hallmark of liver cirrhosis regardless of aetiology [41], the telomeres in NAFLD patients are shorter than in those affected by cryptogenic cirrhosis. In NAFLD, telomere length correlates with the level of hTERT mRNA, while hTERT-independent mechanisms already start to operate in cryptogenic cirrhosis [45].

4. NASH-induced HCC

Although the association between NAFLD and HCC was first observed more than two decades ago, mostly through NASH-induced cirrhosis [11], the molecular events that link NAFLD and HCC are still incompletely understood. Following the general principles of cancerogenesis, HCC in cirrhotic liver develops by dysplasia—carcinoma pathway: from a dysplastic cirrhotic nodule. The process is slow and can last for several decades [34]. The genetic events that are prerequisite for malignant change develop in the background of increased cellular proliferation. Hypothetically, it is possible that the molecular portrait of HCC in DNA, mRNA, microRNA and protein level is different in accordance to the inciting factor of the underlying liver disease. If this is true, specific molecular targets may exist for the diagnostics, prevention or treatment of NASH-induced HCC or HCC arising in diabetic and/or obese patients [10].

The course of HCC that is associated with cryptogenic cirrhosis differs from HCC developing in other clinical settings [46]. HCC also varies by epigenetic signature in accordance to the cause [47].

The risk of hepatocellular carcinoma differs by the aetiology of cirrhosis. To estimate this, a large population-based study was carried out in the United Kingdom. All patients diagnosed with liver cirrhosis were identified, and the results were compared to national cancer registry identifying those diagnosed with HCC. The 10-year cumulative incidence of HCC was 4% in cirrhosis induced by chronic viral hepatitis, 3.2% in cirrhosis due to autoimmune or metabolic (in this study—haemochromatosis, alpha-1-antitrypsin deficiency) diseases, 1.2%

in alcohol-induced cirrhosis and 1.1% in cryptogenic cirrhosis, while the same estimates at 1 year were 1.0, 0.8, 0.3 and 0.3%, respectively. This study has the significant benefit of exploring HCC risk in patients that differ by aetiology of cirrhosis but belong to the same population [37]. Considering patients referred for liver transplantation, the frequency of hepatocellular carcinoma in cryptogenic cirrhosis is lower (8%) than in cirrhosis related to chronic hepatitis B (29%) or C (19%) as reported by Alamo et al. [32]. For the epidemiological estimates of HCC in different liver pathology, see also **Table 2** [37, 38].

The causal distribution of HCC shows geographic variations. Thus, in Canadian patients, 45% of cases were attributable to alcohol-induced cirrhosis, 26% to cryptogenic cirrhosis and 13% to hepatitis C. In patients from Saudi Arabia, 47% of HCC were caused by hepatitis C, 27% by cryptogenic cirrhosis and 21% to hepatitis B [48]. In USA, regarding the HCC cause, 54.9% of cases were induced by HCV, 16.4% by alcohol, 14.1% by NAFLD and 9.5% by HBV [10]. In explanted livers, 81.8% of HCC were associated with viral hepatitis, 9.1% with alcohol-induced liver damage and 9.1% with NAFLD [36].

In the USA, the number of NAFLD-associated HCC cases is annually growing for 9%, if the time span 2004–2009 is evaluated [10]. In Europe, NAFLD-related HCC comprised 35% of all HCC cases in 2010. HCC that is not related to hepatitis B or C is becoming increasingly frequent in Japan as well; however, here, it comprises only 10% of all HCC cases [53]. NASH is responsible for higher percentage of HCC in Western than in Eastern societies [12].

Hepatocellular carcinoma in patients affected by metabolic syndrome has distinct morphology [49]. NAFLD-associated HCC is characterised by larger size [34] and moderate or high differentiation degree [34], showing high differentiation as frequently as in 65% of cases [49]. However, the tumours lack capsule thus confirming the true malignant biological potential [34]. This is an important diagnostic trait considering the association between NAFLD, low-grade HCC [49], and liver adenomatosis [50].

The prognostic estimates are somewhat controversial. The NAFLD-associated hepatocellular carcinomas are diagnosed as more advanced tumours in older patients showing higher cardiovascular morbidity. The patients are less likely to receive liver transplant and have higher

Estimate	Alcohol-induced cirrhosis	Autoimmune and genetic diseases	Chronic hepatitis	Cryptogenic cirrhosis	Reference
SIR; 95% CI	70.6; 59.5–83.2	47.0; ¹ 12.6–120.2	42.7; ² 25.2–67.3	43.4; 30.3–60.4	Sorensen et al. [38]
Incidence rate per 1000 person years; 95% CI	3.2; 2.1–4.8	5.3; 2.6–10.5	7.6; ³ 4.3–13.4	3.1; 1.6–5.9	West et al. [37]

Abbreviations: SIR, standardised incidence ratio; CI, confidence interval.

¹Primary biliary cirrhosis.

²Including viral and autoimmune causes.

³Viral hepatitis.

Table 2. Epidemiological estimates of hepatocellular carcinoma by the cause of chronic advanced liver pathology.

tumour-specific mortality [10]. HCC associated with cryptogenic cirrhosis is larger than cancers related to HCV even in patients who correspond to Milan criteria [51]. However, after curative treatment, the recurrence risk and mortality are lower for HCC arising in cryptogenic cirrhosis—finding that is in accordance with the grade difference [52].

Although previously it was considered that HCC risk is limited to cirrhotic patients, currently at least 25–30% of NAFLD-related hepatocellular carcinomas develop in the absence of cirrhosis [9]. In Japanese group, 33% of NAFLD-related HCC occurred in the background of none or mild fibrosis contrasting with only 16% in alcohol-induced HCC [53]. According to other researchers, up to 65% of NAFLD-associated HCC evolve in the absence of fibrosis [49]. The proportion of NAFLD-associated HCC developing in non-cirrhotic liver has been variably estimated as 15, 38, or 49% [54–57]. These tumours tend to be larger [57].

The development of HCC in noncirrhotic liver has been associated with malignant transformation in liver cell adenoma [34, 49]. Malignant change in hepatic adenoma correlates with metabolic syndrome [58]. Inflammatory molecular type of liver cell adenoma shows clinical correlation with obesity. The underlying molecular basis could include either activated IL-6 signalling or hyperoestrogenemia associated with obesity. However, a controversy exists here as inflammatory type of liver adenoma is not prone to malignisation [50].

Several pathogenetic ways account for a tumour-promoting environment in obesity and diabetes, allowing to distinguish the pathogenesis of HCC linked to NAFLD from that of viral and other aetiologies.

Obesity has been linked to higher frequency of cancers in a variety of tissues [59, 60] including the liver (**Table 3**). HCC is increasingly diagnosed among obese individuals. In a prospective cohort of the Cancer Prevention Study with more than 900,000 North American subjects, the relative risk of dying from liver cancer among men with a body mass index reaching or exceeding 35 kg/m² was remarkably higher (4.5 fold) compared to a reference group with normal body weight. In a large cohort involving 362,552 Swedish men, the relative risk of HCC in individuals with a body mass index reaching or exceeding 30 kg/m² was 3.1 fold higher than in controls having normal weight. Studies from other parts of the world indicate that the link between obesity and increased incidence of HCC has been globally recognised [61].

Obesity has a significant tumour-promoting effect regarding HCC. This effect largely depends on the chronic general low-grade inflammatory response it induces, which involves production of TNF- α and IL-6. Both these molecular mediators are tumour-promoting cytokines [62] and major drivers of cell proliferation in NAFLD and NASH [21]. TNF- α and other mediators produced by activated inflammatory macrophages stimulate compensatory hepatocyte proliferation and expand HCC progenitors. TNF- α further reinforces the inflammatory microenvironment and induces expression of chemokines (CCL2, CCL7 and CXCL13) and growth factors/cytokines (IL-1 β , IL-6, TNF- α itself and hepatocyte growth factor) both by progenitors of hepatocellular carcinoma and surrounding cells [63]. TNF- α up-regulates the cellular proliferation through the molecular pathways of nuclear factor kappaB, mTOR and wide spectrum of kinases. The proliferative and anti-apoptotic activities of IL-6 are largely mediated through the signal transducer and activator of transcription 3, STAT3 [10]. IL-6 also

Location	Level of evidence
Oesophageal adenocarcinoma	Strong
Colorectal cancer in males	Strong
Pancreatic cancer	Strong
Breast cancer	Strong
Endometrial cancer	Strong
Renal cancer	Strong
Multiple myeloma	Strong
Liver cancer	Highly suggestive
Colonic cancer in females	Suggestive
Ovarian cancer	Suggestive
Prostate cancer	Suggestive
Thyroid cancer	Suggestive
Melanoma in males	Weak

Table 3. Obesity-related human cancers [60].

contributes to the metabolic background of cancer sustaining insulin resistance that can be improved by systemic neutralization of IL-6 [64].

Another mechanism involved in the progression of NAFLD to HCC in obese individuals is the imbalance between leptin and adiponectin. Particularly, obesity is linked to increased levels of leptin [34]. Apart from its role in obesity-associated insulin resistance and inflammation, leptin is a pro-inflammatory, pro-angiogenic, and pro-fibrogenic cytokine with a growth-promoting effect by activating the Janus kinase/STAT, phosphoinositide 3-kinase (PI3K)/Akt, and extracellular signal-regulated kinase (ERK) signalling pathways [61]. The up-regulation of PI3K/Akt pathway leads to activation of downstream molecular mediator mTOR that is found in 40% of HCC cases. Leptin-induced up-regulation of mTOR also inhibits autophagy—a process that normally would limit oxidative stress by removing damaged mitochondria. Suppression of autophagy, in turn, increases oxidative tissue damage and subsequent inflammation [21]. Since leptin exerts pro-inflammatory and pro-fibrogenic effects by activating Kupffer cells and stellate cells, it has been associated to disease progression in fibrotic NAFLD [10]. Leptin can also promote invasion and migration of hepatocellular carcinoma cells [65].

Adiponectin, another major adipokine with potent anti-inflammatory, antiangiogenic and tumour growth-limiting properties, is suppressed in obesity [15, 24]. Adiponectin activates 5'-adenosine monophosphate-activated protein kinase, which can suppress tumour growth and increase apoptosis by regulating the mTOR and c-Jun N-terminal kinase/caspase 3 pathways. Moreover, adiponectin opposes the effects of leptin by inhibiting activation of Akt and STAT3, as well as by increasing the expression of SOCS3: the suppressor of cytokine signalling 3 [61].

Thus, low adiponectin levels may be insufficient to suppress endotoxin-mediated inflammatory signalling in Kupffer cells and other macrophages, as well as control angiogenesis, a pivotal mechanism of tumour growth [10]. Microarray analysis of tissue adiponectin levels in HCC patients revealed that adiponectin expression was inversely correlated with tumour size, supporting the hypothesis that adiponectin may inhibit proliferation and dedifferentiation [66].

HCC can show marked accumulation of fat within the neoplastic cells (**Figure 4**). In a study by Salomao et al., 36% of patients who developed HCC in the setting of steatohepatitis were diagnosed as having a steatohepatitic variant of HCC as compared to 1.3% of HCC patients without steatohepatitis [67]. Increased intensity of fatty acid synthesis and characteristic pattern of perilipin proteins has been demonstrated in HCC. Regarding gene expression pattern, activated lipogenesis is associated with higher cell proliferation and worse prognosis in HCC [10]. Hypothetically, HCC cells might benefit from the energetic value of fat compounds or use lipids as building blocks of new cells.

Lipotoxicity, defined as the cellular dysfunction caused by ectopic deposition of fat in non-adipose tissues, may contribute to the development of HCC in NAFLD. Activated oxidation of fatty acids generates high burden of free radicals and lipid peroxide compounds that oxidise and damage large molecules and cell organoids, e.g., mitochondria and endoplasmic reticulum. The damaged cells are subjected to apoptosis, leading to higher activity in liver destruction and progression towards cirrhosis that in turn is closely associated with enhanced proliferation and accumulation of genetic damage. Accumulation of fatty acids may interfere with cellular signalling and promote oncogenesis through altered regulation of gene transcription [10]. Oxidative stress can induce mutations in the tumour suppressor gene *TP53* in a pattern observed in HCC [68].

Adipose tissue expansion, release of pro-inflammatory cytokines, and lipotoxicity collectively promote systemic and hepatic insulin resistance, resulting in hyperinsulinemia [34]. The risk of HCC in patients affected by diabetes mellitus is 2.31 [57]. Insulin resistance and hyperinsulinemia are the most common metabolic features of NAFLD, which correlate with impaired hepatic clearance of insulin and have been linked to tumour development [69]. Deregulated metabolic effects of insulin result in excessive activation of proliferative signalling cascades.

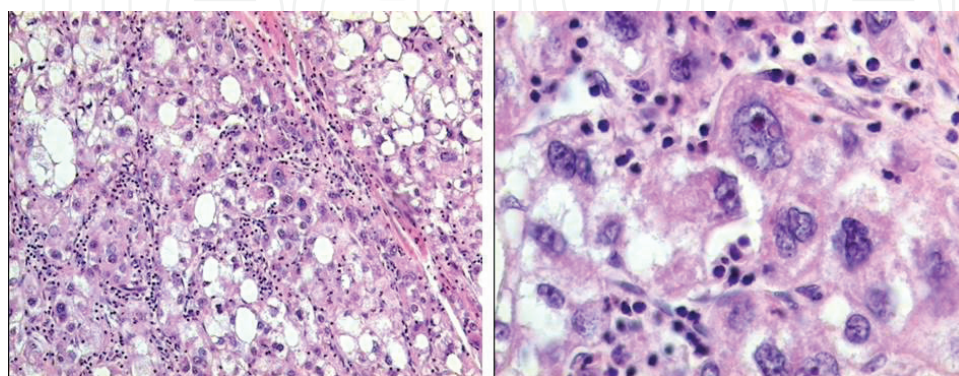


Figure 4. Hepatocellular carcinoma showing nuclear atypia and presence of fat in tumour cells. Haematoxylin-eosin stain, original magnification 100× and 400×.

Hyperinsulinemia causes reduced hepatic synthesis of insulin-like growth factor (IGF)-binding protein-1 and increased bioavailability of IGF1, which further promotes cellular proliferation and inhibits apoptosis [10, 34]. It has been shown recently that elevated fasting insulin, which is inversely related to insulin sensitivity, is an independent risk factor for HCC. Baseline serum levels of C-peptide have also been found to be associated with a higher risk of HCC in the general population independently of obesity and other established liver cancer risk factors [69]. Loss of heterozygosity for IGF2 has been observed in over 60% of HCC cases. This likely coincides with IGF2 overexpression, found in HCC, which has been associated to reduced apoptosis and increased cellular proliferation [68].

The importance of insulin resistance is illustrated by the observations that obesity and type 2 diabetes mellitus comprise increased HCC risk even regardless of the presence or cause of liver cirrhosis [9].

A number of studies have demonstrated a critical role for phosphatase and tensin homolog (PTEN) in the progression of NASH to tumour. *PTEN* deletion results in PKB/Akt activation, promoting proliferation and reducing apoptosis. Insulin-like growth factor 2 mRNA binding protein p62 was reported to be a possible upstream regulator of PTEN. Aberrant microRNAs contribute to carcinogenesis. MiR-21 was found to be another upstream regulator of PTEN participating in NASH-associated cancer induction [10, 14, 70].

The oral iron test has revealed increased absorption of iron compounds in patients affected by NASH [71]. In turn, increased amount of iron in liver tissues is associated with increased risk of HCC in patients affected by NASH-related liver cirrhosis [72]. As the reductive conversion of Fe(III) to Fe(II) necessitates increased oxidation of other compounds, oxidative DNA damage can develop and lead to the malignancy [34, 73]. Iron overload also is known to enhance insulin resistance [74] and to act in concert with other factors damaging liver. The significance of iron overload in hepatic carcinogenesis is shown in several models. The risk of HCC is increased in hereditary haemochromatosis, characterised by excessive iron accumulation in the body and caused by excessive absorption because of homozygous C282Y mutation in *HFE* gene. Almost 8–10% of patients with hereditary haemochromatosis develop HCC. Increased relative risk of HCC (10×) has also been demonstrated in association with long-lasting excess dietary iron intake [37, 74, 75]. Thus, there is significant evidence of the carcinogenic action of iron overload, and evidence of iron accumulation in NAFLD and especially NASH that allows drawing conclusion that iron metabolites are contributing to the development of NASH-related HCC.

The expression profile of *Wnt* signalling genes in NASH strongly suggests inhibition of Wnt pathway. IHC staining of β -catenin shows predominately membrane staining with loss of nuclear staining indicating that β -catenin is not active in NASH. In contrast, 20–90% of HCC cases exhibit active Wnt pathway [76]. Thus, the long-lasting conversion of NASH into HCC hypothetically involves up-regulation of Wnt pathway either by activators or loss of inhibitors [77].

Hepatocyte apoptosis is a prominent feature of NASH (**Figure 5**). The executing mechanism of apoptosis includes activation of characteristic lytic enzymes—the caspases. In an apoptotic

hepatocyte, activated caspase-3 is splitting various cell structures, including cytokeratin (CK) 18—the intermediary filament that represents the specific cytoskeleton protein of hepatocytes. Consequently, blood tests can reveal increased concentration of CK18 fragments [70]. In liver tissues, CK8 and CK18-containing Mallory bodies are evident by light microscopy as large, brightly eosinophilic inclusions in liver cell cytoplasm. Although Mallory hyaline is the hallmark of alcohol-induced hepatitis, its development can also be induced by diet rich in saturated fatty acids. The molecular pathways associated with Mallory body development include IL-6, protein p62 that binds ubiquitin in cell cytoplasm, and reduced concentration of HSP72 that prevents protein misfolding. The presence of CK18 in Mallory bodies correlates with plasma CK18 levels [78]. In a longitudinal paired liver biopsy study, the change of CK18 correlated with disease progression. Patients with increased NAFLD activity score 3 years after initial evaluation had greater increase of plasma CK18 compared with those who had stable or decreased activity score [79]. El-Zefzafy et al. proved that CK18 was a sensitive indicator of the severity of liver disease and also could predict the development of HCC. In their study, the sensitivity and specificity of serum CK18 were 95 and 96.7%, respectively, with a cut-off value of 534.5 U/L for HCC diagnosis [80].

In a study by Salomao et al., devoted to HCC in NASH, immunohistochemically there was diffuse loss of cytoplasmic CK8/18 and an increased number of activated hepatic stellate cells within the steatohepatic HCC, identical to the pattern seen in the surrounding non-neoplastic liver [67, 81].

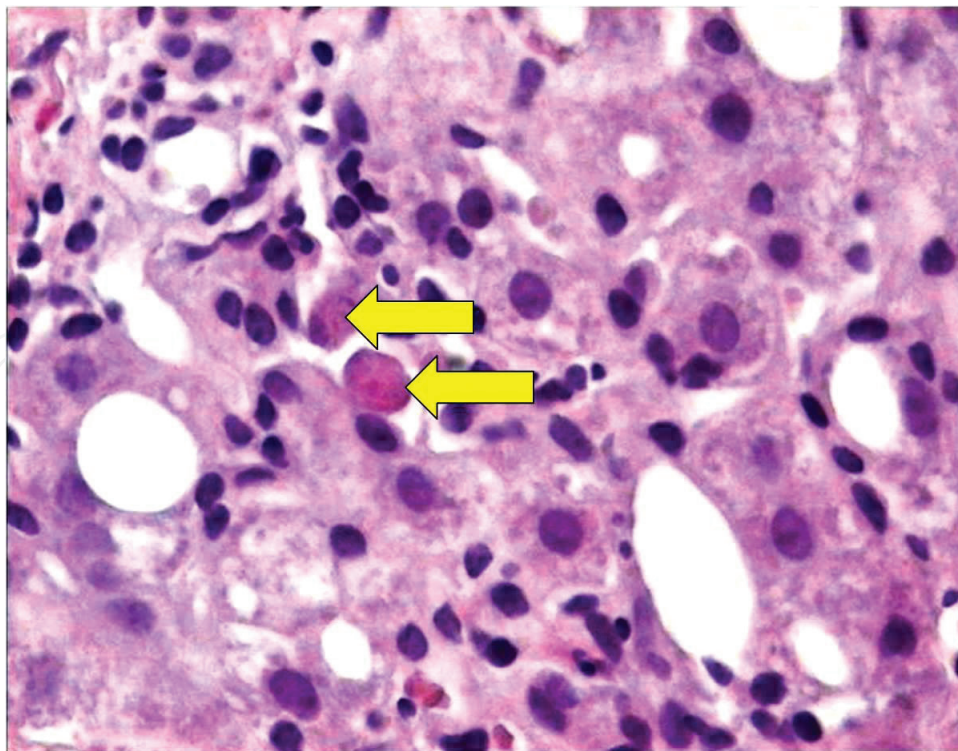


Figure 5. Apoptotic bodies (arrows) in non-alcoholic steatohepatitis. Haematoxylin-eosin stain, original magnification 400×.

The HCC development shows complex associations with telomere shortening. The senescence-associated secretory program of liver stellate cells promotes carcinogenesis. The telomere shortening induces also genomic instability thus facilitating HCC development [41]. Indeed, HCC is characterised by significantly shorter telomeres in comparison to adjacent tissues [82]. However, cancer cells still maintain unlimited proliferation. Evidently, hepatocellular carcinoma cells develop compensatory mechanisms either for telomere extension or for cellular proliferation despite telomere shortening. The elongation of telomeres again can be ensured via diverse mechanisms, including hTERT or alternative lengthening of telomeres via telomerase-independent mechanism seen in 7% of HCC cases [41].

Over the progression of HCC, the telomere length changes in contrary direction. Early liver carcinogenesis is associated with telomere shortening, while disease progression is associated with telomere extension, cell immortalisation and reactivation of telomerase [83]. Longer telomeres in HCC are associated with higher stage (regional or distant spread *versus* localised tumour) and grade (III–IV *versus* lower grade) as well as with worse survival [83, 84]. Telomerase promotes HCC development via several pathways, not limited to maintenance of telomeres and thus cellular proliferation. In addition, hTERT can act as a transcription factor in the Wnt molecular cascade [41]. Experimental data by HCC induction in telomerase-deficient mice have shown increased number of early tumours and reduced incidence of high-grade HCC [85].

Interestingly, shorter telomeres are observed more frequently (telomere length ratio between HCC and surrounding tissues lower than the mean, 70.1% *versus* higher, 29.9%) in HCC that is not related to hepatitis B (50.0% *versus* 50.0%) or C (60.0% *versus* 40.0%), or alcohol abuse (50.0% *versus* 50.0%), although the difference does not reach statistical significance [83]. Telomere shortening can be detected in peripheral blood. Notably, this assay can be used to predict HCC persistence (by telomere shortening) in cases attributable to viral hepatitis B or C but not in HCC attributable to non-infectious causes despite comparable size of patient groups [86].

Genetic predisposition has been studied in NAFLD trying to identify those patients that are at particularly increased risk of HCC. The possible candidate genes could be associated with telomere length and mechanisms involved in preserving telomeres [42]. About 10% of patients affected both by HCC and NASH have germline mutations in *hTERT* in comparison to complete absence of such mutations in NASH patients having cirrhosis and healthy controls [41]. In addition, *PNPLA3* polymorphisms have been studied in NAFLD patients, finding twice increased risk of HCC in association with rs738409 C>G. The proposed mechanism involves retinol metabolism in hepatic stellate cells [34].

The interaction of these pathogenetic mechanisms and genetic predisposition finally results in the increased incidence of HCC in NAFLD that reaches 76–201 per 100,000 contrasting with the incidence of 4.9–16 per 100,000 of the general population [57].

5. Potential treatment strategies

As no specific treatment is approved for NAFLD, lifestyle interventions play the leading role in NAFLD management. Weight loss due to low calorie diet in combination with physical activities is the main therapeutic approach in overweight patients with NAFLD. As hypertriglyceridemia is a frequent and promoting feature of NAFLD [87] reduction of the triglyceride

level must be among therapeutic goals. In severe hypertriglyceridemia, total fat consumption should be limited to less than 30 g/day, and carbohydrate amount in daily nutrition should be strictly controlled as well [88].

Physical activity has beneficial effect of reducing triglyceride level, even independently from diet [89]. Thus, at least 30 min of moderate activity most days of the week would be a necessary part of dyslipidemia management [90]. Loss of 5% of body weight decreases hepatic steatosis, but body weight loss of 10% could even improve inflammation and fibrosis in liver [87].

Experimentally investigating hepatocyte-specific PTEN-deficient mouse model, Piguet et al. showed that physical activity could reduce HCC growth in fatty liver. In PTEN-deficient mice, HCC incidence was 71% of exercised mice and 100% of sedentary mice. In addition, liver tumour volume in exercised mice was significantly smaller than that of sedentary mice (444 ± 551 versus 945 ± 1007 mm³) [91]. The physiological substantiation relies on fact that regular physical activity could inhibit mTOR complex, which is engaged in cell growth and proliferation [92].

Increased hepatic free cholesterol accumulation is typical for NASH. Statins are commonly prescribed to reduce cholesterol synthesis in the liver and thus serum levels of free cholesterol [14]. In a recent European multi-centre cohort study, statin use was associated with protection from steatosis (odds ratio, OR 0.09; 95% CI, 0.01–0.32; $p = 0.004$), steatohepatitis (OR, 0.25; 95% CI, 0.13–0.47; $p < 0.001$), and fibrosis stage F2–F4 (OR, 0.42; 95% CI, 0.20–0.80; $p = 0.017$). The protective effect of statins on steatohepatitis was stronger in subjects not carrying the I148M PNPLA3 risk variant ($p = 0.02$), indicating the role of genetic predisposition [93]. Statins also have been associated with reduced risk (range, 0.46–0.79) of HCC [94].

In a meta-analysis, including 4298 patients with HCC, statin use was associated with a 37% reduction in the risk of hepatocellular carcinoma. The effect was stronger in Asian patients but was also present in Western populations. Moreover, the reduction of cancer risk was independent of statin lipid-lowering effects [95]. Several hypotheses have been proposed, including statin ability to inhibit cell proliferation via inhibition of v-myc avian myelocytomatosis viral oncogene homolog protein phosphorylation which seems to play a role in liver carcinogenesis [96], as well as capacity to inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase, which activates multiple proliferative pathways [95]. Simvastatin selectively induces apoptosis in cancer, but not in healthy cells. This proapoptotic effect is maintained via RAF/MAPK1/ERK and growth-inhibitory action by suppression of angiogenesis and proteasome pathway [95, 96]. However, data about liver carcinogenesis and statin effects remain controversial. In another large meta-analysis, including 86,936 participants, no beneficial effect of statin in terms of incidence or death from cancer was observed. Even more, in 67,258 patients who received statins, 35 new liver cancers and 24 deaths from liver cancer were reported showing no significant difference from control group, comprising 67,279 patients who received placebo, and developed 33 new liver cancer ($p = 0.93$) cases leading to 24 deaths ($p = 1.00$) as analysed by Carrat [97].

Metformin, a widely prescribed drug for treating type 2 diabetes mellitus, is one of the most extensively recognised metabolic modulators which decreases aminotransferase levels and hepatic insulin resistance. It has no beneficial effects on NAFLD histology but still retains an

important anti-cancer action [87, 98]. The hypothetic antitumor mechanisms of metformin are believed to be (1) inhibition of mTOR, (2) weight loss and (3) suppressed production of ROS and the associated DNA damage, in combination with (4) reduction of hyperinsulinemia, which is known to lead to cell proliferation [99]. In meta-analysis comprising 105,495 patients with type 2 diabetes, Zhang et al. showed that metformin was associated with an estimated 70% reduction in the risk of developing HCC [98]. The risk reduction in metformin users is significant, regarding both incidence (78%) and mortality (77%) from HCC [100].

The mammalian target of rapamycin (mTOR) promotes growth in a majority of liver cancers, including hepatocellular carcinoma. It participates in the formation of two protein complexes—mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin and has ability to activate downstream targets which regulate cellular growth and metabolism. Prolonged mTORC1 activation is related to liver steatosis and insulin resistance in obese patients [14, 101]. Due to the ability suppress mTORC1, rapamycin and its analogues Everolimus and Temsirolimus have been tested to treat HCC. Unfortunately, results have not been promising. In a phase 3 study of patients with advanced HCC, Everolimus increased the frequency of hepatic injury and showed no improvements regarding survival [14]. After 2 weeks with rapamycin treatment, the lipid droplets in the liver decreased, as well as ROS burden. However, rapamycin treatment promoted liver damage with augmented IL-6 and decreased anti-inflammatory IL-10 production, leading to increased hepatic inflammation and hepatocyte necrosis [101].

Inflammation promotes development of complications in patients with cirrhosis contributing to mortality and to liver insufficiency mediated by pro-inflammatory cytokines. The most recognisable pro-inflammatory cytokine associated with liver damage in case of NAFLD is TNF- α that can be inhibited by pentoxifylline. Lebrec et al. performed randomised, placebo controlled, double-blind trial assessing pentoxifylline effect in 335 patients with cirrhosis. Although pentoxifylline had no effect on short-term mortality, it significantly ($p = 0.04$) prolonged the complication-free time span [102].

Knowing the important role of NADPH oxidases (NOXs) and production of ROS in liver fibrosis, different strategies to prevent the oxidative damage have been developed [23]. In hepatocytes, NOX4 mediates suppressor effects on TGF- β and can inhibit hepatocyte growth and liver carcinogenesis. In turn, dual NOX4/NOX1 pharmacological inhibitor GKT137831 could decrease both the apparition of fibrogenic markers as well as hepatocyte apoptosis *in vivo* [103].

Currently, multikinase inhibitor sorafenib is the only pharmacological agent that prolongs survival of HCC patients, although the median survival is improved only by 12 weeks [14]. It acts against Raf-1 and B-raf, vascular endothelial growth factor (VEGF) receptors and platelet-derived growth factor receptor kinases [104]. Sorafenib as well as VEGF inhibitors have radiosensitizing effect. However, combined regimens including sorafenib and liver stereotactic radiation or whole liver radiotherapy are characterized by poor tolerability [104]. Various beneficial effects of sorafenib have been reported in liver cirrhosis. As epithelial-mesenchymal transition and TGF- β play crucial roles in liver fibrosis, Ma et al. proved that sorafenib had ability to strikingly suppress TGF- β 1 induced epithelial-mesenchymal transition, as well as apoptosis in hepatic stellate cells, in dose-dependent manner [105].

Several treatment strategies might involve the telomere and telomerase complex. In cancer, telomerase inhibitors might arrest tumour growth, prevent further malignisation in surrounding cirrhotic nodules and/or enhance HCC chemosensitivity. In early liver disease, telomerase activation might prevent tissue loss if the etiologic factor cannot be removed. This could be reached via transplantation of liver cells engineered for hTERT expression, direct supply of hTERT to the patient's cells or by small molecules enhancing telomerase activity. However, side effects and enhanced cancer risk must be considered and prevented [41]. The treatment modulating cellular senescence and proliferation control may also target p21 [106–108] and p53 [109] pathways.

The p21 protein, a strong and universal inhibitor of cyclin-dependant kinases, is an important regulator of cell proliferation, apoptosis and senescence [107, 108]. Based on its intracellular location and the molecular background, it can have dual activity. Intranuclear p21 acts as tumour suppressor, as it binds cyclin-dependant kinases and thus suppresses cellular proliferation. Cytoplasmic p21 prevents apoptosis by binding caspases and promotes proliferation and migration of p53-deficient cells. The p21 pathway is also closely associated with senescence. Few small molecular inhibitors of p21 are known, including LLW10, butyrolactone and UC2288. In addition, sorafenib also exhibits anti-p21 activity. LLW10 binds to p21 and induces proteosomal degradation via ubiquitination. Despite the reliable mechanism, the high concentration that is necessary for sufficient activity as well as the instability of LLW10 prevents it from being clinically useful drug. Butyrolactone also induces proteosomal degradation of p21. UC2288 decreases p21 concentration via suppressed transcription and modified posttranscriptional modulation [107]. In turn, up-regulation of p21 can be achieved via statins or by anticancer agents including histone deacetylase inhibitors [106]. Induction of senescence would be desirable if the tumour is already present while suppressed senescence might prevent or slow down the development of liver cirrhosis. As was noted, it is possible to modulate p21 level in both directions. However, the net effects must be carefully considered and studied experimentally, knowing the bidirectional activity of p21.

p21 is also an effector of p53-mediated responses in cells maintaining functional p53. In p53-deficient cell, it manifests carcinogenic effects. Thus, restoration of wild-type p53 could be attractive, either in combination with p21-targeted treatment or with other oncological approach. In liver cancer, restoration of p53 activity has resulted in senescence and increased immune response. The therapeutic approaches could include (1) restoration of wild type function to mutant p53 by low molecular weight compounds PRIMA 1 or PRIMA-1MET. The last one has progressed to phase II clinical trials; (2) stabilising p53 due to blocked interaction with MDM2 or MDM4 by nutlins, representing low molecular weight molecules, or by stapled peptides; (3) gene therapy using viral vectors that has already been tested in HCC; (4) induction of synthetic lethality [109].

6. Conclusions

Non-alcoholic steatohepatitis is recognised as the cause of NASH-induced cirrhosis. It has also been associated with a significant fraction of cases previously diagnosed as cryptogenic

cirrhosis. Liver cirrhosis can become further complicated by hepatocellular carcinoma, the most frequent primary liver tumour known for serious prognosis and limited treatment options. In addition, the development of HCC in NAFLD patients can precede cirrhosis in a significant fraction of cases. NAFLD is the major hepatic manifestation of obesity and associated metabolic diseases, such as diabetes mellitus. With increasing prevalence of these conditions, NAFLD has become the most common liver disorder worldwide. It affects around 25% of general population and 90% of patients suffering from morbid obesity, i.e., having body mass index equal or greater than 40 kg/m².

The mechanisms of liver steatosis include up-regulation of inflammatory cytokines, as TNF- α , IL-6 and CCL2, released from extrahepatic adipose tissues due to prolonged low-grade inflammation triggered by hypoxia-induced death of fast-growing fat cells. Insulin resistance further contributes to NAFLD and can be aggravated by the pro-inflammatory cytokine background. Free fatty acids and cholesterol cause lipotoxicity due to released reactive oxygen species as well as toxic metabolites generated by non-oxidative biochemical pathways. Decreased level of adiponectin, exaggerated oxidative stress and hepatic iron accumulation also are among the mechanisms of NAFLD.

In the pathogenesis of NAFLD, 20–30% of patients, initially affected by simple liver steatosis, develop hepatic inflammation and thus correspond to the diagnostic criteria of NASH. These cases are at risk to progress to liver cirrhosis and hepatocellular carcinoma. The standardised incidence ratio of HCC in NASH patients reaches 4.4. Regarding the epidemiological profile of hepatocellular carcinoma, the proportion of NASH-related cases is growing and has increased from 8.3 to 13.5% in the time period 2002–2012.

Obesity has been linked to higher frequency of cancers in different organs including the liver. The relative risk of HCC-attributable death in obese patients (body mass index equal or greater than 35 kg/m²) can be as high as 4.5. The underlying mechanisms of carcinogenesis include chronic general low-grade inflammation characterised by elevated levels of TNF- α and IL-6, both of which are tumour-promoting cytokines and major drivers of cell proliferation in NAFLD and NASH. The increased levels of leptin and suppressed production of adiponectin represent another mechanism involved in the progression of NAFLD to HCC in obese individuals. Leptin is a pro-inflammatory, pro-angiogenic and pro-fibrogenic cytokine with a growth-promoting effect. Adiponectin has anti-inflammatory, antiangiogenic and tumour growth-limiting properties. Insulin resistance and hyperinsulinemia lead to excessive cell proliferation. Iron compound deposition has also been related to HCC development in NAFLD-related cirrhosis, possibly due to oxidative DNA damage. Thus, the same molecular pathways that induced NAFLD continue to be active until the development of HCC. These mechanisms are supplemented by critical genetic events including *PTEN* deletion, switch from inactivated to upregulated Wnt pathway and typical mutation pattern in *TP53*. Certain microRNAs, including miR-21, act as molecular switches.

Pathogenetically related molecular markers, e.g., cytokeratin 18, can serve as predictive tests to detect increased risk of HCC.

The molecular pathogenesis of NAFLD is closely related to the selection of treatment targets. NAFLD patients can benefit from low calorie diet, reducing hypertriglyceridemia and potentially reversing steatosis and even fibrosis; physical activity inhibiting mTOR complex;

statins influencing cholesterol synthesis, RAF/MAPK1/ERK and p21 pathway; metformin acting through suppression of mTOR and ROS; pentoxifyllin lowering production of pro-inflammatory cytokines. Multikinase inhibitor sorafenib is indicated in HCC patients. Bidirectional regulation of telomere attrition, senescence, and p21 pathway could be at least theoretically considered in the future. Restoration of wild-type p53 activity becomes possible. The regulation of miRNA machinery also represents a highly attractive future treatment option.

Thus, NAFLD is gaining increasing importance in nowadays medicine as a frequent condition that can lead to such grave complications as liver cirrhosis and hepatocellular carcinoma. Awareness of the molecular profile is helpful to identify the treatment targets and predictive markers.

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References

- [1] Tandler DA. Pathogenesis of nonalcoholic fatty liver disease. UpToDate [Internet] Mar 2016 [Updated: Mar 09, 2016]. Available from: <http://www.uptodate.com/contents/pathogenesis-of-nonalcoholic-fatty-liver-disease> [Accessed: Mar 03, 2017]
- [2] White DL, Kanwal F, El-Serag HB. Association between nonalcoholic fatty liver disease and risk for hepatocellular cancer, based on systematic review. *Clinical Gastroenterology and Hepatology*. 2012;**10**(12):1342-1359.e2. DOI: 10.1016/j.cgh.2012.10.001
- [3] El-Serag HB, Kanwal F. Epidemiology of hepatocellular carcinoma in the United States: Where are we? Where do we go? *Hepatology*. 2014;**60**(5):1767-1775. DOI: 10.1002/hep.27222

- [4] Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nature Reviews Gastroenterology and Hepatology*. 2013;**10**(11):656-665. DOI: 10.1038/nrgastro.2013.183
- [5] Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease. Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016;**64**(1):73-84. DOI: 10.1002/hep.28431
- [6] Marengo A, Jouness RI, Bugianesi E. Progression and natural history of nonalcoholic fatty liver disease in adults. *Clinical Liver Disease*. 2016;**20**(2):313-324. DOI: 10.1016/j.cld.2015.10.010
- [7] Fingas CD, Best J, Sowa JP, Canbay A. Epidemiology of nonalcoholic steatohepatitis and hepatocellular carcinoma. *Clinical Liver Disease*. 2016;**8**(5):119-122. DOI: 10.1002/cld.585
- [8] Younossi ZM, Otgonsuren M, Henry L, Venkatesan C, Mishra A, Erario M, Hunt S. Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009. *Hepatology*. 2015;**62**(6):1723-1730. DOI: 10.1002/hep.28123
- [9] Pais R, Barritt AS 4th, Calmus Y, Scatton O, Runge T, Lebray P, et al. NAFLD and liver transplantation: Current burden and expected challenges. *Journal of Hepatology*. 2016;**65**(6):1245-1257. DOI: 10.1016/j.jhep.2016.07.033
- [10] Baffy G, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: An emerging menace. *Journal of Hepatology*. 2012;**56**(6):1384-1391. DOI: 10.1016/j.jhep.2011.10.027
- [11] Sheth SG, Sanjiv C. Natural history and management of nonalcoholic fatty liver disease in adults. UpToDate [Internet]. Nov 2016 [Updated: Nov 08, 2016]. Available from: <http://www.uptodate.com/contents/natural-history-and-management-of-nonalcoholic-fatty-liver-disease-in-adults> [Accessed: Mar 03, 2017]
- [12] Agrawal S, Duseja AK. Non-alcoholic fatty liver disease: East versus West. *Journal of Clinical and Experimental Hepatology*. 2012;**2**(2):122-134. DOI: 10.1016/S0973-6883(12)60101-7
- [13] Day CP, James OF. Steatohepatitis: A tale of two "hits"? *Gastroenterology*. 1998;**114**(4):842-845
- [14] Charrez B, Qiao L, Hebbard L. Hepatocellular carcinoma and non-alcoholic steatohepatitis: The state of play. *World Journal of Gastroenterology*. 2016;**22**(8):2494-2502. DOI: 10.3748/wjg.v22.i8.2494
- [15] Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. *Hepatology*. 2010;**52**(5):1836-1846. DOI: 10.1002/hep.24001
- [16] Wong RJ, Cheung R, Ahmed A. Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. *Hepatology*. 2014;**59**(6):2188-2195. DOI: 10.1002/hep.26986

- [17] Hao C, Zhu PX, Yang X, Han ZP, Jiang JH, Zong C, et al. Overexpression of SIRT1 promotes metastasis through epithelial-mesenchymal transition in hepatocellular carcinoma. *BMC Cancer*. 2014;**14**:978. DOI: 10.1186/1471-2407-14-978
- [18] Schwartz JM, Carithers RL. Clinical features and diagnosis of primary hepatocellular carcinoma. UpToDate [Internet]. Dec 2016 [Updated: Dec 20, 2016]. Available from: <https://www.uptodate.com/contents/clinical-features-and-diagnosis-of-primary-hepatocellular-carcinoma> [Accessed: Mar 03, 2017]
- [19] Polyzos SA, Toulis KA, Goulis DG, Zavos C, Kountouras J. Serum total adiponectin in nonalcoholic fatty liver disease: A systematic review and meta-analysis. *Metabolism*. 2011;**60**(3):313-326. DOI: 10.1016/j.metabol.2010.09.003
- [20] Wang Y, Liu Z, Zou W, Hong H, Fang H, Tong W. Molecular regulation of miRNAs and potential biomarkers in the progression of hepatic steatosis to NASH. *Biomarkers in Medicine*. 2015;**9**(11):1189-1200. DOI: 10.2217/bmm.15.70
- [21] Kolb R, Sutterwala FS, Zhang W. Obesity and cancer: Inflammation bridges the two. *Current Opinion in Pharmacology*. 2016;**29**:77-89. DOI: 10.1016/j.coph.2016.07.005
- [22] Cusi K. Role of insulin resistance and lipotoxicity in non-alcoholic steatohepatitis. *Clinical Liver Disease*. 2009;**13**(4):545-563. DOI: 10.1016/j.cld.2009.07.009
- [23] Friedman SL. Pathogenesis of hepatic fibrosis. UpToDate [Internet]. Nov 24, 2015 [Updated: Nov 24, 2015]. Available from: <http://www.uptodate.com/contents/pathogenesis-of-hepatic-fibrosis> [Accessed: Mar 03, 2017]
- [24] Sharifnia T, Antoun J, Verriere TG, Suarez G, Wattacheril J, Wilson KT et al. Hepatic TLR4 signaling in obese NAFLD. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. 2015;**309**(4):G270-278. DOI: 10.1152/ajpgi.00304.2014
- [25] Novo E, Busletta C, Bonzo LV, Povero D, Paternostro C, Mareschi K, et al. Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. *Journal of Hepatology*. 2011;**54**(5):964-974. DOI: 10.1016/j.jhep.2010.09.022
- [26] Pietrangelo A. Iron in NASH, chronic liver diseases and HCC: How much iron is too much? *Journal of Hepatology*. 2009;**50**(2):249-251. DOI: 10.1016/j.jhep.2008.11.011
- [27] Zimmermann A, Zimmermann T, Schattenberg J, Pöttgen S, Lotz J, Rossmann H, et al. Alterations in lipid, carbohydrate and iron metabolism in patients with non-alcoholic steatohepatitis (NASH) and metabolic syndrome. *European Journal of Internal Medicine*. 2011;**22**(3):305-310. DOI: 10.1016/j.ejim.2011.01.011
- [28] Seki E, Brenner DA. Recent advancement of molecular mechanisms of liver fibrosis. *Journal of Hepato-Biliary-Pancreatic Sciences*. 2015;**22**(7):512-518. DOI: 10.1002/jhbp.245
- [29] Trautwein C, Friedman SL, Schuppan D, Pinzani M. Hepatic fibrosis: Concept to treatment. *Journal of Hepatology*. 2015;**62**(1 Suppl):S15-24. DOI: 10.1016/j.jhep.2015.02.039

- [30] Mercado-Irizarry A, Torres EA. Cryptogenic cirrhosis: Current knowledge and future directions. *Clinical Liver Disease*. 2016;**7**(4):69-72. DOI: 10.1002/cld.539
- [31] Rinaldi L, Nascimbeni F, Giordano M, Masetti C, Guerrera B, Amelia A, et al. Clinical features and natural history of cryptogenic cirrhosis compared to hepatitis C virus-related cirrhosis. *World Journal of Gastroenterology*. 2017;**23**(8):1458-1468. DOI: 10.3748/wjg.v23.i8.1458
- [32] Alamo JM, Bernal C, Barrera L, Marin LM, Suarez G, Serrano J, et al. Liver transplantation in patients with cryptogenic cirrhosis: Long term follow-up. *Transplantation Proceedings*. 2011;**43**(6):2230-2232. DOI: 10.1016/j.transproceed.2011.05.017
- [33] Hashemi SJ, Hajiani E, Masjedizadeh A, Makvandi M, Shayesteh AA, Alavinejad SP, et al. Occult hepatitis B infection in patients with cryptogenic liver cirrhosis in southwest of Iran. *Jundishapur Journal of Microbiology*. 2015;**8**(3):e16873. DOI: 10.5812/jjm.16873
- [34] Margini C, Dufour JF. The story of HCC in NAFLD: From epidemiology, across pathogenesis, to prevention and treatment. *Liver International*. 2016;**36**(3):317-324. DOI: 10.1111/liv.13031
- [35] Michitaka K, Nishiguchi S, Aoyagi Y, Hiasa Y, Tokumoto Y, Onji M; Japan Etiology of Liver Cirrhosis Study Group. Etiology of liver cirrhosis in Japan: A nationwide survey. *Journal of Gastroenterology*. 2010;**45**(1):86-94. DOI: 10.1007/s00535-009-0128-5
- [36] Nayak NC, Jain D, Vasdev N, Gulwani H, Saigal S, Soin A. Etiologic types of end-stage chronic liver disease in adults: Analysis of prevalence and their temporal changes from a study on native liver explants. *European Journal of Gastroenterology & Hepatology*. 2012;**24**(10):1199-1208. DOI: 10.1097/MEG.0b013e32835643f1
- [37] West J, Card TR, Aithal GP, Fleming KM. Risk of hepatocellular carcinoma among individuals with different aetiologies of cirrhosis: A population-based cohort study. *Alimentary Pharmacology & Therapeutics*. 2017;**45**(7):983-990. DOI: 10.1111/apt.13961
- [38] Sorensen HT, Friis S, Olsen JH, Thulstrup AM, Mellemejaer L, Linet M, et al. Risk of liver and other types of cancer in patients with cirrhosis: A nationwide cohort study in Denmark. *Hepatology*. 1998;**28**(4):921-925
- [39] Singal AK, Hasanin M, Kaif M, Wiesner R, Kuo YF. Nonalcoholic steatohepatitis is the most rapidly growing indication for simultaneous liver kidney transplantation in the United States. *Transplantation*. 2016;**100**(3):607-612. DOI: 10.1097/TP.0000000000000945
- [40] Kong D, Zhang F, Zhang Z, Lu Y, Zheng S. Clearance of activated stellate cells for hepatic fibrosis regression: Molecular basis and translational potential. *Biomedicine & Pharmacotherapy*. 2013;**67**(3):246-250. DOI: 10.1016/j.biopha.2012.10.002
- [41] Donati B, Valenti L. Telomeres, NAFLD and chronic liver disease. *International Journal of Molecular Sciences*. 2016;**17**(3):383. DOI: 10.3390/ijms17030383

- [42] Carulli L, Anzivino C. Telomere and telomerase in chronic liver disease and hepatocarcinoma. *World Journal of Gastroenterology*. 2014;**20**(20):6287-6292. DOI: 10.3748/wjg.v20.i20.6287
- [43] Calado RT, Brudno J, Mehta P, Kovacs JJ, Wu C, Zago MA, et al. Constitutional telomerase mutations are genetic risk factors for cirrhosis. *Hepatology*. 2011;**53**(5):1600-1607. DOI: 10.1002/hep.24173
- [44] Hartmann D, Srivastava U, Thaler M, Kleinhans KN, N'kontchou G, Scheffold A, et al. Telomerase gene mutations are associated with cirrhosis formation. *Hepatology*. 2011;**53**(5):1608-1617. DOI: 10.1002/hep.24217
- [45] Laish I, Mannasse-Green B, Hadary R, Biron-Shental T, Konikoff FM, Amiel A, Kitay-Cohen Y. Telomere dysfunction in nonalcoholic fatty liver disease and cryptogenic cirrhosis. *Cytogenetic and Genome Research*. 2016;**150**(2):93-99. DOI: 10.1159/000454654
- [46] Siriwardana RC, Niriella MA, Dassanayake AS, Liyanage C, Gunathilaka B, Jayathunge S, de Silva HJ. Clinical characteristics and outcome of hepatocellular carcinoma in alcohol related and cryptogenic cirrhosis: A prospective study. *Hepatobiliary & Pancreatic Diseases International*. 2015;**14**(4):401-405
- [47] Hlady RA, Tiedemann RL, Puszyk W, Zendejas I, Roberts LR, Choi JH, et al. Epigenetic signatures of alcohol abuse and hepatitis infection during human hepatocarcinogenesis. *Oncotarget*. 2014;**5**(19):9425-9443. DOI: 10.18632/oncotarget.2444
- [48] Alsohaibani F, Porter G, Al-Ashgar H, Walsh M, Berry R, Molinari M, Peltekian KM. Comparison of cancer care for hepatocellular carcinoma at two tertiary-care referral centers from high and low endemic regions for viral hepatitis. *Journal of Gastrointestinal Cancer*. 2011;**42**(4):228-235. DOI: 10.1007/s12029-010-9200-x
- [49] Paradis V, Zalinski S, Chelbi E, Guedj N, Degos F, Vilgrain V, et al. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: A pathological analysis. *Hepatology*. 2009;**49**(3):851-859. DOI: 10.1002/hep.22734
- [50] Chang CY, Hernandez-Prera JC, Roayaie S, Schwartz M, Thung SN. Changing epidemiology of hepatocellular adenoma in the United States: Review of the literature. *International Journal of Hepatology* 2013;**2013**:604860. DOI: 10.1155/2013/604860
- [51] Feng J, Wu J, Zhu R, Feng D, Yu L, Zhang Y, et al. Simple risk score for prediction of early recurrence of hepatocellular carcinoma within the Milan criteria after orthotopic liver transplantation. *Scientific Reports*. 2017;**7**:44036. DOI: 10.1038/srep44036
- [52] Takuma Y, Nouse K, Makino Y, Gotoh T, Toshikuni N, Morimoto Y, et al. Outcomes after curative treatment for cryptogenic cirrhosis-associated hepatocellular carcinoma satisfying the Milan criteria. *Journal of Gastroenterology and Hepatology*. 2011;**26**(9):1417-1424. DOI: 10.1111/j.1440-1746.2011.06775.x

- [53] Kimura T, Kobayashi A, Tanaka N, Sano K, Komatsu M, Fujimori N, et al. Clinicopathological characteristics of non-B non-C hepatocellular carcinoma without past hepatitis B virus infection. *Hepatology Research*. 2017; **47**(5):405-418. DOI: 10.1111/hepr.12762
- [54] Tokushige K, Hashimoto E, Horie Y, Taniai M, Higuchi S. Hepatocellular carcinoma in Japanese patients with nonalcoholic fatty liver disease, alcoholic liver disease, and chronic liver disease of unknown etiology: Report of the nationwide survey. *Journal of Gastroenterology*. 2011; **46**(10):1230-1237. DOI: 10.1007/s00535-011-0431-9
- [55] Yasui K, Hashimoto E, Komorizono Y, Koike K, Arai S, Imai Y, et al. Characteristics of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma. *Clinical Gastroenterology and Hepatology*. 2011; **9**(5):428-433. DOI: 10.1016/j.cgh.2011.01.023
- [56] Duan XY, Qiao L, Fan JG. Clinical features of nonalcoholic fatty liver disease-associated hepatocellular carcinoma. *Hepatobiliary & Pancreatic Diseases International*. 2012; **11**(1): 18-27. DOI: 10.1016/S1499-3872(11)60120-3
- [57] Leung C, Yeoh SW, Patrick D, Ket S, Marion K, Gow P, Angus PW. Characteristics of hepatocellular carcinoma in cirrhotic and non-cirrhotic non-alcoholic fatty liver disease. *World Journal of Gastroenterology*. 2015; **21**(4):1189-1196. DOI: 10.3748/wjg.v21.i4.1189
- [58] Farges O, Ferreira N, Dokmak S, Belghiti J, Bedossa P, Paradis V. Changing trends in malignant transformation of hepatocellular adenoma. *Gut*. 2011; **60**(1):85-89. DOI: 10.1136/gut.2010.222109
- [59] Hirabayashi S. The interplay between obesity and cancer: A fly view. *Disease Models & Mechanisms*. 2016; **9**(9):917-926. DOI: 10.1242/dmm.025320
- [60] Kyrgiou M, Kalliala I, Markozannes G, Gunter MJ, Paraskevaides E, Gaba H, et al. Adiposity and cancer at major anatomical sites: Umbrella review of the literature. *BMJ*. 2017; **356**:j477. DOI: 10.1136/bmj.j477
- [61] Karagozian R, Derdák Z, Baffy G. Obesity-associated mechanisms of hepatocarcinogenesis. *Metabolism*. 2014; **63**(5):607-617. DOI: 10.1016/j.metabol.2014.01.011
- [62] Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell*. 2010; **140**(2):197-208. DOI: 10.1016/j.cell.2009.12.052
- [63] Nakagawa H, Umemura A, Taniguchi K, Font-Burgada J, Dhar D, Ogata H, et al. ER stress cooperates with hypernutrition to trigger TNF-dependent spontaneous HCC development. *Cancer Cell*. 2014; **26**(3):331-343. DOI: 10.1016/j.ccr.2014.07.001
- [64] Catrysse L, van Loo G. Inflammation and the metabolic syndrome: The tissue-specific functions of NF- κ B. *Trends in Cell Biology*. 2017; **27**(6):417-429. DOI: <http://dx.doi.org/10.1016/j.tcb.2017.01.006>
- [65] Saxena NK, Sharma D, Ding X, Lin S, Marra F, Merlin D, Anania FA. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Research*. 2007; **67**(6):2497-2507. DOI: 10.1158/0008-5472.CAN-06-3075

- [66] Dalamaga M, Diakopoulos KN, Mantzoros CS. The role of adiponectin in cancer: A review of current evidence. *Endocrine Reviews*. 2012;**33**(4):547-594. DOI: 10.1210/er.2011-1015
- [67] Salomao M, Remotti H, Vaughan R, Siegel AB, Lefkowitz JH, Moreira RK. The steatohepatitic variant of hepatocellular carcinoma and its association with underlying steatohepatitis. *Human Pathology*. 2012;**43**(5):737-746. DOI: 10.1016/j.humpath.2011.07.005
- [68] Page JM, Harrison SA. NASH and HCC. *Clinical Liver Disease*. 2009;**13**(4):631-647. DOI: 10.1016/j.cld.2009.07.007
- [69] Chettouh H, Lequoy M, Fartoux L, Vigouroux C, Desbois-Mouthon C. Hyperinsulinaemia and insulin signalling in the pathogenesis and the clinical course of hepatocellular carcinoma. *Liver International*. 2015;**35**(10):2203-2217. DOI: 10.1111/liv.12903
- [70] Yu J, Shen J, Sun TT, Zhang X, Wong N. Obesity, insulin resistance, NASH and hepatocellular carcinoma. *Seminars in Cancer Biology*. 2013;**23**(6 Pt B):483-491. DOI: 10.1016/j.semcancer.2013.07.003
- [71] Hoki T, Miyanishi K, Tanaka S, Takada K, Kawano Y, Sakurada A, et al. Increased duodenal iron absorption through up-regulation of divalent metal transporter 1 from enhancement of iron regulatory protein 1 activity in patients with nonalcoholic steatohepatitis. *Hepatology*. 2015;**62**(3):751-761. DOI: 10.1002/hep.27774
- [72] Sorrentino P, D'Angelo S, Ferbo U, Micheli P, Bracigliano A, Vecchione R. Liver iron excess in patients with hepatocellular carcinoma developed on non-alcoholic steatohepatitis. *Journal of Hepatology*. 2009;**50**(2):351-357. DOI: 10.1016/j.jhep.2008.09.011
- [73] Fargion S, Valenti L, Fracanzani AL. Role of iron in hepatocellular carcinoma. *Clinical Liver Disease*. 2014;**3**(5):108-110. DOI: 10.1002/cld.350
- [74] Kew MC. Hepatic iron overload and hepatocellular carcinoma. *Liver Cancer*. 2014;**3**(1):31-40. DOI: 10.1159/000343856
- [75] Sikorska K, Bernat A, Wroblewska A. Molecular pathogenesis and clinical consequences of iron overload in liver cirrhosis. *Hepatobiliary & Pancreatic Diseases International*. 2016;**15**(5):461-479. DOI: 10.1016/S1499-3872(16)60135-2
- [76] Thompson MD, Monga SP. WNT/beta-catenin signaling in liver health and disease. *Hepatology*. 2007;**45**(5):1298-1305. DOI: 10.1002/hep.21651
- [77] Clarke JD, Novak P, Lake AD, Shipkova P, Aranibar N, Robertson D, et al. Characterization of hepatocellular carcinoma related genes and metabolites in human nonalcoholic fatty liver disease. *Digestive Diseases and Sciences*. 2014;**59**(2):365-374. DOI: 10.1007/s10620-013-2873-2879
- [78] Takaki A, Kawai D, Yamamoto K. Molecular mechanisms and new treatment strategies for non-alcoholic steatohepatitis (NASH). *International Journal of Molecular Sciences*. 2014;**15**(5):7352-7379. DOI: 10.3390/ijms15057352

- [79] Wong VW, Wong GL, Choi PC, Chan AW, Li MK, Chan HY, et al. Disease progression of non-alcoholic fatty liver disease: A prospective study with paired liver biopsies at 3 years. *Gut*. 2010;**59**(7):969-974. DOI: 10.1136/gut.2009.205088
- [80] El-Zefzafy W, Eltokhy H, Mohamed NA, Abu-Zahab Z. Significance of serum cytokeratin-18 in prediction of hepatocellular carcinoma in chronic hepatitis C infected Egyptian patients. *Open Access Macedonian Journal of Medical Sciences*. 2015;**3**(1):117-123. DOI: 10.3889/oamjms.2015.021
- [81] Knudsen ES, Gopal P, Singal AG. The changing landscape of hepatocellular carcinoma: etiology, genetics, and therapy. *American Journal of Pathology*. 2014;**184**(3):574-583. DOI: 10.1016/j.ajpath.2013.10.028
- [82] Zhang Y, Shen J, Ming-Whei, Lee YP, Santella RM. Telomere length in hepatocellular carcinoma and paired adjacent non-tumor tissues by quantitative PCR. *Cancer Investigation*. 2007;**25**(8):668-677
- [83] Lee HW, Park TI, Jang SY, Park SY, Park WJ, Jung SJ, Lee JH. Clinicopathological characteristics of TERT promoter mutation and telomere length in hepatocellular carcinoma. *Medicine (Baltimore)*. 2017;**96**(5):e5766. DOI: 10.1097/MD.0000000000000576
- [84] Yang B, Shebl FM, Sternberg LR, Warner AC, Kleiner DE, Edelman DC, et al. Telomere length and survival of patients with hepatocellular carcinoma in the United States. *PLoS One*. 2016;**11**(11):e0166828. DOI: 10.1371/journal.pone.0166828
- [85] Farazi PA, Glickman J, Jiang S, Yu A, Rudolph KL, DePinho RA. Differential impact of telomere dysfunction on initiation and progression of hepatocellular carcinoma. *Cancer Research*. 2003;**63**(16):5021-5027
- [86] Feng W, Yu D, Li B, Luo OY, Xu T, Cao Y, Ding Y. Paired assessment of liver telomere lengths in hepatocellular cancer is a reliable predictor of disease persistence. *Bioscience Reports*. 2017;**37**(2):pii: BSR20160621. DOI: 10.1042/BSR20160621
- [87] Lonardo A, Bellentani S, Argo CK, Ballestri S, Byrne CD, et al. Non-alcoholic Fatty Liver Disease Study Group, Epidemiological modifiers of non-alcoholic fatty liver disease: Focus on high-risk groups. *Digestive and Liver Disease*. 2015;**47**(12):997-1006. DOI: 10.1016/j.dld.2015.08.004
- [88] European Association for Cardiovascular Prevention & Rehabilitation, Reiner Z, Catapano AL, De Backer G, Graham I, Taskinen MR, et al. ESC/EAS Guidelines for the management of dyslipidaemias: The Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). *European Heart Journal*. 2011;**32**(14):1769-1818. DOI: 10.1093/eurheartj/ehr158
- [89] Vanhees L, Geladas N, Hansen D, Kouidi E, Niebauer J, Reiner Z, et al. Importance of characteristics and modalities of physical activity and exercise in the management of cardiovascular health in individuals with cardiovascular risk factors: Recommendations

from the EACPR. Part II. *European Journal of Preventive Cardiology*. 2012;**19**(5):1005-1033. DOI: 10.1177/1741826711430926

- [90] Corey KE, Chalasani N. Management of dyslipidemia as a cardiovascular risk factor in individuals with nonalcoholic fatty liver disease. *Clinical Gastroenterology and Hepatology*. 2014;**12**(7):1077-1084. DOI: 10.1016/j.cgh.2013.08.014
- [91] Piguet AC, Saran U, Simillion C, Keller I, Terracciano L, Reeves HL, Dufour JF. Regular exercise decreases liver tumors development in hepatocyte-specific PTEN-deficient mice independently of steatosis. *Journal of Hepatology*. 2015;**62**(6):1296-1303. DOI: 10.1016/j.jhep.2015.01.017
- [92] Degasperi E, Colombo M. Distinctive features of hepatocellular carcinoma in non-alcoholic fatty liver disease. *The Lancet Gastroenterology & Hepatology*. 2016;**1**(2):156-164. DOI: 10.1016/S2468-1253(16)30018-8
- [93] Dongiovanni P, Petta S, Mannisto V, Mancina RM, Pipitone R, Karja V, et al. Statin use and non-alcoholic steatohepatitis in at risk individuals. *Journal of Hepatology*. 2015;**63**(3):705-712. DOI: 10.1016/j.jhep.2015.05.006
- [94] El-Serag HB, Johnson ML, Hachem C, Morgana RO. Statins are associated with a reduced risk of hepatocellular carcinoma in a large cohort of patients with diabetes. *Gastroenterology*. 2009;**136**(5):1601-1608. DOI: 10.1053/j.gastro.2009.01.053
- [95] Singh S, Singh PP, Singh AG, Murad MH, Sanchez W. Statins are associated with a reduced risk of hepatocellular cancer: A systematic review and meta-analysis. *Gastroenterology*. 2013;**144**(2):323-332. DOI: 10.1053/j.gastro.2012.10.005
- [96] Nouredin M, Rinella ME. Nonalcoholic fatty liver disease, diabetes, obesity, and hepatocellular carcinoma. *Clinical Liver Disease*. 2015;**19**(2):361-379. DOI: 10.1016/j.cld.2015.01.012
- [97] Carrat F. Statin and aspirin for prevention of hepatocellular carcinoma: What are the levels of evidence? *Clinics and Research in Hepatology and Gastroenterology*. 2014;**38**(1):9-11. DOI: 10.1016/j.clinre.2013.09.007
- [98] Zhang ZJ, Zheng ZJ, Shi R, Su Q, Jiang Q, Kip KE. Metformin for liver cancer prevention in patients with type 2 diabetes: A systematic review and meta-analysis. *The Journal of Clinical Endocrinology & Metabolism*. 2012;**97**(7):2347-2353. DOI: 10.1210/jc.2012-1267
- [99] Jara JA, López-Muñoz R. Metformin and cancer: Between the bioenergetic disturbances and the antifolate activity. *Pharmacological Research*. 2015;**101**:102-108. DOI: 10.1016/j.phrs.2015.06.014
- [100] Zhang P, Li H, Tan X, Chen L, Wang S. Association of metformin use with cancer incidence and mortality: A meta-analysis. *Cancer Epidemiology*. 2013;**37**(3):207-218. DOI: 10.1016/j.canep.2012.12.009

- [101] Umemura A, Park EJ, Taniguchi K, Lee JH, Shalapour S, Valasek MA, et al. Liver damage, inflammation, and enhanced tumorigenesis after persistent mTORC1 inhibition. *Cell Metabolism*. 2014;**20**(1):133-144. DOI: 10.1016/j.cmet.2014.05.001
- [102] Lebrec D, Thabut D, Oberti F, Perarnau JM, Condat B, Barraud H, et al. Pentoxifylline does not decrease short-term mortality but does reduce complications in patients with advanced cirrhosis. *Gastroenterology*. 2010;**138**(5):1755-1762. DOI: 10.1053/j.gastro.2010.01.040
- [103] Crosas-Molist E, Fabregat I. Role of NADPH oxidases in the redox biology of liver fibrosis. *Redox Biology*. 2015;**6**:106-111. DOI: 10.1016/j.redox.2015.07.005
- [104] Goody RB, Brade AM, Wang L, Craig T, Brierley J, Dinniwell R, et al. Phase I trial of radiation therapy and sorafenib in unresectable liver metastases. *Radiotherapy & Oncology*. 2017;**123**(2):234-239. DOI: 10.1016/j.radonc.2017.01.018
- [105] Ma R, Chen J, Liang Y, Lin S, Zhu L, Liang X, Cai X. Sorafenib: A potential therapeutic drug for hepatic fibrosis and its outcomes. *Biomedicine & Pharmacotherapy*. 2017;**88**:459-468. DOI: 10.1016/j.biopha.2017.01.107
- [106] Abbas T, Dutta A. p21 in cancer: Intricate networks and multiple activities. *Nature Reviews Cancer*. 2009;**9**(6):400-414. DOI: 10.1038/nrc2657
- [107] Liu R, Wettersten HI, Park SH, Weiss RH. Small-molecule inhibitors of p21 as novel therapeutics for chemotherapy-resistant kidney cancer. *Future Medicinal Chemistry*. 2013;**5**(9):991-994. DOI: 10.4155/fmc.13.56
- [108] Georgakilas AG, Martin OA, Bonner WM. p21: A two-faced genome guardian. *Trends in Molecular Medicine*. 2017;**23**(4):310-319. DOI: 10.1016/j.molmed.2017.02.001
- [109] Duffy MJ, Synnott NC, McGowan PM, Crown J, O'Connor D, Gallagher WM. p53 as a target for the treatment of cancer. *Cancer Treatment Reviews*. 2014;**40**(10):1153-1160. DOI: 10.1016/j.ctrv.2014.10.004